"A STUDY ON BACTERIAL, FUNGAL AND PARASITIC AGENTS IN INFECTIOUS KERATITIS PATIENTS DUE TO TRAUMA IN A TERTIARY CARE OPHTHALMIC HOSPITAL"

Dissertation submitted to

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In partial fulfillment of the requirements for the degree of

BRANCH – IV – M.D. DEGREE (MICROBIOLOGY)

APRIL 2013.

CERTIFICATE

This is to certify that the dissertation entitled **"A STUDY ON BACTERIAL, FUNGAL AND PARASITIC AGENTS IN INFECTIOUS KERATITIS PATIENTS DUE TO TRAUMA IN A TERTIARY CARE OPHTHALMIC HOSPITAL"** is the bonafide work done by **Dr.C.SENTHIL VADIVU,** during her M.D. Degree Branch – IV (Microbiology) is a bonafide research work carried out by her under the direct supervision & guidance.

Prof.V.KANAGASABAI
The Dean
Madras Medical College &
Rajiv Gandhi Govt. General Hospital,
Chennai – 3

DR.G.JAYALAKSHMI.,M.D
Director,
Institute of Microbiology,
Madras Medical College,
Chennai- 600 003.

DECLARATION

I, **Dr.C.SENTHILVADIVU**, declare that, I carried out this, work on **"A STUDY ON BACTERIAL, FUNGAL AND PARASITIC AGENTS IN INFECTIOUS KERATITIS PATIENTS DUE TO TRAUMA IN A TERTIARY CARE OPHTHALMIC HOSPITAL"** at the Institute of Microbiology, Madras Medical College, I also declare that this bonafide work or a part of this work was not submitted by me or any other for any award, degree or diploma to any other University, Board, either in India or abroad.

This is submitted to the TamilNadu Dr.M.G.R.Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D Degree examination in Microbiology.

Place : Chennai **Dr. C.SENTHILVADIVU**

Date :

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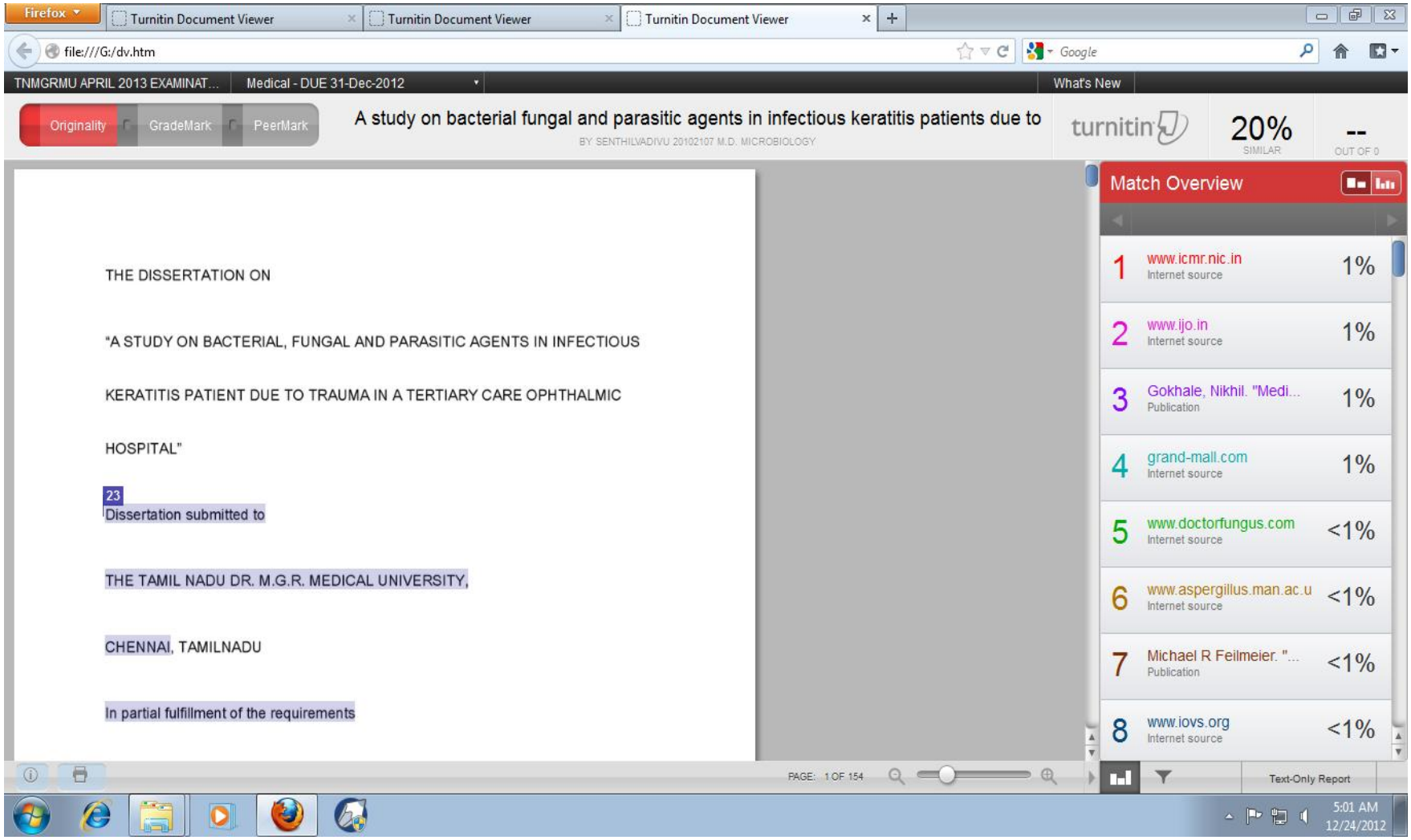
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INTRODUCTION

INTRODUCTION

Inflammation of the cornea (keratitis) is characterized by corneal oedema, cellular infiltration and associated conjunctival reaction.

Corneal infection or infectious keratitis is one of the most important cause of preventable blindness in the developing world. Suppurative keratitis (corneal ulceration) occurs frequently subsequent to corneal injury. Delay in diagnosing the nature of infection is one of the paramount factors, which is responsible for inappropriate initial therapy and poor outcomes. Trauma maybe initiated by air-borne particles and is especially common in agricultural workers. Vegetable matter such as rice husks, soil, sand or dust, getting into the eye can cause damage to the ocular surface. Other risk factors include contact lens wearer, contamination of topical medications, lid abnormalities and ocular surface diseases such as dry eye.

The reported incidence range from 11 per 1,00,000 person years in the United States to 799 per 1,00,000 person years in the developing nations like Nepal. In India the annual incidence is

reported to be 11.3 per 10,000. Infectious keratitis requires prompt diagnosis and treatment to prevent blindness or even enucleation.

Left untreated, or treated inappropriately, the patient can go blind in the affected eye. Corneal ulceration is an important cause of ocular morbidity. The scarring of the cornea when the ulcer heals can lead to significant visual impairment.

Infectious keratitis may be caused by bacteria, fungi, viruses or protozoa. A detailed work up is necessary to arrive at a proper diagnosis and to initiate appropriate treatment.

Considering the importance of corneal ulceration and its impact on vision, the present study was conducted to identify the aetiological agents and their susceptibility profiles in patients attending a tertiary care Regional Institute of Ophthalmology and Rajiv Gandhi Government General hospital in Chennai.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Corneal ulceration in the developing world is a silent epidemic. Corneal infection is a leading cause of ocular morbidity and blindness worldwide.[56]

Structure of the cornea:

The cornea consists of five layers namely:

1. The epithelium
2. Bowman's membrane
3. Substantia propria or stroma
4. Descemet's membrane
5. The endothelium

Nutrition of the Cornea

Cornea is an avascular structure. It derives nutrition from:

1. Perilimbal blood vessels: Anterior ciliary vessels invade the periphery of the cornea (limbus) for about 1 mm.
2. Aqueous humor: It supplies glucose and other nutrients by process of simple diffusion or active transport.
3. Oxygen from atmospheric air is derived directly through the tear film.[3]

Nerve Supply

The nerve supply is purely sensory. It is derived from the ophthalmic division of the 5th cranial nerve through the nasociliary branch.[3]

Functions

Two primary functions of the cornea are.

1. It acts as a major refracting medium.
2. It protects the intraocular contents.

This is possible by maintaining corneal transparency and replacement of its tissues. Transparency is maintained by:

i. Regular arrangement of corneal lamellae (lattice theory of cornea)
ii. Avascularity
iii. Relative state of dehydration.[3]

DISEASES OF THE CORNEA

Disease of the cornea are of clinical importance as they often leave permanent opacities, which lowers the visual acuity and the associated complications may even lead to blindness.[56]

1. Inflammations (Kera`titis)

- ❖ Bacterial keratitis
- ❖ Fungal keratitis
- ❖ Viral keratitis
- ❖ Parasitic keratitis

2. Degenerations

a) Arcus senilis

b) Arcus juvenilis

c) Band-shaped keratopathy

d) Hereditary corneal dystrophy

e) Reis-Bucklers' dystrophy

f) Endothelial corneal dystrophy of Fuchs

3. Ectasias

a) Keratoconus

b) Keratoglobus

4. Pigmentations

a) Blood Staining

b) Argyrosis

c) Kayser-Fleisher's ring

Inflammations of the Cornea

Inflammations of the cornea (keratitis) is characterized by corneal oedema, cellular infiltration and associated conjunctival reaction.[3]

1. **Exogenous infection** e.g. *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, E.coli, Proteus spp, Klebsiella spp, H.influenzae,* etc. common. Usually organisms in the conjunctival sac, lacrimal sac (dacryocystitis), infected foreign body, etc. cause inflammation of the cornea.
2. **From the ocular tissue**
    a. Conjunctival diseases spread to the epithelium.
    b. Scleral diseases spread to the stroma.
    c. Uveal tract diseases spread to the endothelium.
3. **Endogenous infection** – It is usually due to hypersensitivity reaction.

Predisposing Factors

Keratitis due to microbial aetiology (Bacterial, Fungal, Viral and Parasitic) has the following predisposing factor. The following predisposing factors are the same for Bacterial, Fungal, Viral and Parasitic agents.[3,67]

1. Epithelial damage due to trauma, e.g. minute foreign body, misdirected eyelash, vegetative matter such as husks soil, sand, dust, air-borne particle.
2. Virulent organisms, e.g. *Streptococcus pneumoniae, Pseudomonas spp, Neisseria gonorrohoae,* etc.
3. Poor resistance
    - Xerosis and keratomalacia (vitamin A deficiency)
    - Protein calorie malnutrition
    - Corneal oedema leads to desquamation of epithelium
    - Neuroparalytic keratitis, e.g. Herpes zoster, leprosy
    - Exposure of the cornea due to proptosis, facial nerve palsy.[67]

Cesar *et al,* in 2008 from UK reported that trauma is the leading predisposing factor in Infectious keratitis [13] .

M.J. Bharathi *et al*,in 2003 from South India has reported that the epidemiology and etiology of bacterial keratitis is specific to the region. Screening patients for predisposing factors, treating the co-existing ocular diseases, and educating them about proper lens care and risk of infection may reduce the occurrence of bacterial keratitis[9].

B.H.Jeng *et al*,in 2003 from UK has concluded that risk factors for infectious keratitis included contact lens use (55%), ocular surface disease (16.6%), trauma (11.9%), and bullous keratopathy (1.3%)[47].

Reema nath *et al* from Upper Assam in 2011 has concluded that injury with vegetative matter as the most common risk factor[81].

M.Srinivasan *et al* in 1997 from Madurai studied that corneal injury (65.4%) was the major predisposing factor in the aetiology of Infectious keratitis[94].

Sadia Seth *et al* from Peshawar in 2010 has reported that ocular trauma was the most common cause found in 39% of patients[84].

Youhanna HW Ibrahuin *et al* in 2009 from UK as has concluded that wearing of the contact lens was the main predisposing factor (31%)in infectious keratitis patients [109].

Many studies have reported well recognized association of contact lens wear with Acanthamoeba keratitis and fungal keratitis [97].

Ocular trauma particularly with vegetative matter is a well known predisposing factor in fungal keratitis[54].

Dry, dusty and windy environment have a increased risk of microtrauma to the cornea, resulting in an increasing incidence of fungal keratitis during these seasons[77].

Stages of Corneal Ulcer

Stages of Corneal Ulcer are categorised into 3 stages namely[3]

1. Progressive stage
    - There is grey zone of infiltration by polymorphs.
    - Localised necrosis and sloughing of sequestrum is present.
    - Saucer-shaped ulcer with overhanging edges due to oedema is characteristic.

2. Regressive stage
    - The dead material is thrown off and the oedema subsides.
    - The floor and edges of the ulcer are smooth and transparent.
3. Healing stage
    - Minute superficial vessels grow in from the limbus near the ulcer.
    - There is formation of fibrous tissue which fills the gap. The irregular arrangement of fibrous tissue results in opacity, as the new fibres refract the light irregularly. As Bowman's membrane never regenerates, permanent opacity remains, if it is damaged.

Symptoms

1. Pain - Cornea is richly supplied by ophthalmic division of the trigeminal nerve.
2. Photophobia – There is undue sensitivity to light.
3. Impairment of visual acuity occurs due to corneal opacity.
4. Lacrimation – There is excessive reflex tear production.

Signs

1. Blepharospasm – There is tight closure of the eyelids specially in children.
2. Corneal opacification occurs due to infiltration and oedema.
3. Ciliary congestion with conjunctival hyperaemia is present.
4. Hypopyon or pus in the anterior chamber may be present.

COMPLICATIONS

1. Corneal opacity

2. Ectatic cicatrix (Keratectasia)

3. Descemetocele (Keratocele)

4. Perforation

EPIDEMIOLOGY

Dr. Rajan K. Anand in 2010 from Bihar studied that corneal ulcer is a common vision threatening condition among the rural population, next only to cataract. The annual incidence of corneal ulcer in India is reported to be 11.3 per 10,0000[80].

Singh SK *et al* from Nepal in 2011 studied that the incidence of corneal ulceration in Nepal is one of the highest reported in the world. The Bhaktapur Eye study revealed it to be 799 per 100,000

population per year. (Upadhyay *et al*, 2001) which is seven times higher than in South India (Gonzales *et al* , 1996) and seventy times greater than that reported in the USA (Erie JC *et al*, 1993)[93].

M.Srinivasan *et al* in 1997 from Madurai has concluded increased incidence of infectious keratitis in males (65%)[94].

Sadia Seth *et al* in 2010 from Peshawar (India) studied that the incidence of microbial keratitis was high in males (67%)[84].

Youhanna HW Ibrahim *et al* in 2009 from UK has concluded predominance of corneal ulcer in female (54%)[109].

B.H. Jeng *et al* in 2003 in US studied that the highest rate of infectious keratitis was found in females (63%)[47,48].

Males are more commonly affected than females, but in the agricultural population the incidence may be equal or more in females[50].

PATHOGENESIS

Adherence of microbes to cornea

↓

Invasion into corneal stroma

↓

Inflammation and neovascularisation

↓

Interruption of the host immune response

↓

Stromal degradative process[3]

Reichert R *et al* in 1984 studied that the adherence of *S.aureus, S.pneumoniae and Pseudomonas spp,* to ulcerated corneal epithelium is significantly higher than other bacteria and may account in part for their frequent isolation[82].

Feilmeier, Michael R *et al* from Nepal in 2010 studied that smear microscopy is reliable in determining the etiology of the keratitis and can be used to help guide initial therapy in this setting[26].

Acanthamoeba species enter through minor abrasions in the cornea produced by contact lens or external injury. In the cornea it elicits inflammation with hypopyon formation. Further progression of infection leads to perforation[75].

BACTERIAL KERATITIS

Bacterial keratitis is a loss in the continuity of the corneal epithelium associated with tissue infiltration and necrosis.[3]

Etiology

Bacterial keratitis is always an exogenous infections are common due to pyogenic organisms which invade the cornea from outside such as *Staphylococcus spp, Streptococcus pneumoniae, Pseudomonas spp, E.coli,* etc.[103]

The common causative bacterial organisms of corneal ulcer are as follows:

i. Gram-positive cocci – *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus hemolyticus, S.pneumoniae.*

ii. Gram-negative cocci – *Neisseria gonorrheae, N. meningitides.*

iii. Gram-positive bacilli – *Nocardia asteroides, Corynebacterium diphtheriae.*

iv. Gram-negative bacilli – *Pseudomonas aeruginosa, Proteus spp, Klebsiella spp, Moraxella spp,Hemophilus spp, Escherichia coli,* etc.

v. Mycobacteria – *Mycobacterium tuberculosis, M.leprae.*

Three pathogens can invade normal intact epithelium:

i. Neisseria Gonorrhoeae

ii. Neisseria Meningitides

iii. *Corynebacterium Diphtheriae*

There has also been a change in the spectrum of bacteria causing keratitis with time[88]. *Staphylococcus spp, Pseudomonas spp and Streptococcus spp* appear to be the predominant causes of bacterial corneal ulcer in United States[37].

Similarly common bacterial pathogens in most of the studies in India are *Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas spp*[65].

Although relatively uncommon, keratitis caused by *Acinetobacter spp* and *Serratia marcescens* have been documented[60].

FUNGAL KERATITIS

Etiology:

Fungal keratitis is commonly caused by *Candida albicans, Aspergillus fumigatus, Fusarium spp, Pencillium spp, Acremonium spp, curvularia spp, Bipolaris,* etc.[3]

Among the filamentous fungi, *Aspergillus spp is the most common followed by Fusarium spp.* Which are most prevalent in agricultural areas.

Candida albicans is the commonly affecting yeast like fungas in the immunocompromised host.[3]

Incidence:

Fungal keratitis is common in rural agricultural areas, and usually occurs due to ocular trauma involving vegetable matter, e.g. thorn, sharp wooden stick, wheat and paddy husk, branches of tree, etc.[37]

Predisposing Factors

Addition to the common predisposing factor responsible for keratitis are same as for bacterial keratitis

Indiscriminate use of topical or systemic steroids alters host defence mechanism and hence fungal keratitis is found to be more common in immunocompromised host.[51]

Symptoms

Pain, Photophobia, Impairment of vision, Lacrimation are commonly seen. But they are less prominent than equal-sized bacterial ulcer.The presence of yellow patch in the cornea is mainly seen in the fungal keratitis.[3]

Signs

1. A typical lesion is a yellow-white coloured ulcer with indistinct margin, with minimum vascularization.

2. Fungal keratitis is dry in appearance with small satellite lesions around the ulcer due to the stromal infiltration with delicate feathery, finger-like hyphate edges protruding into adjacent stroma.

3. Ulcer margin is often elevated above the surface.

4. Massive hypopyon is present commonly, which is dense and organized.

5. Slit-lamp examination – Endothelial plaque and immune ring may be seen around the ulcer. Some degree of iridocyclitis is usually present.

Jayahar bharathi *et al* in 2007 from south India studied that the incidence of fungal keratitis (66%) was more with agricultural workers where as the bacterial keratitis (57%) was more common in non - agricultural workers[5].

Jagadish chander *et al,* from Chandigarh in 2008, reported that the prevalent organisms involved in microbial keratitis were *Aspergillus spp. (41.18%), Fusarium spp (27%), Candida spp (8.82%), Curvularia spp(5.88%) and Bipolaris spp (5.88%)*[40].

Boucier T *et al,* in 2003 from US has concluded that the most common causative organisms are bacteria although fungi and protists are also pathogens[12].

M.Srinivasan *et al,* from Madurai in 1997 has concluded that *S.pneumoniae* (44.3%) was the predominant organism followed by *Pseudomonas spp* and the most common fungal pathogen isolated was *Fusariuim spp* (47. 1%) followed by *Aspergillus spp*. (16.1%)[94].

Usha Arora *et al*,in 2009 from Amristar has reported that *Aspergillus spp* was the most common isolate followed by *Fusariuim spp, Penicillium spp* and *Curvularia spp*[102].

Species of *Penicillium, Alternaria, Curvularia, Bipolaris, Acremonium, Aureobasidium* were isolated frequently in studies conducted in various parts of India and Nepal[15,52].

Feilmeir *et al,* in 2010 from Nepal has reported that fungal organisms are the most common cause of infectious keratitis inpatient population. *Aspergillus spp* (35%) among fungus and *S.pneumoniae* among bacteria were the most common organisms responsible for keratitis[26].

Lai1a Aktar *et al*, in 2009 from Bangladesh studied that *Pseudomonas spp (24%), S.pneumoniae (17%), Aspergillus spp (13%), Fusariuim spp (7 %) and Curvularia spp (6%)* were found as pathogens causing suppurative corneal ulcer[58].

Mycotic corneal ulceration although not reported as a major cause of keratitis in developed countries is an important cause of ulcerative keratitits in tropical regions including India[83].

Species of *Aspergillus genus* especially *A.fumigatus, A.flavus and A.niger* were the predominate fungal pathogens in studies conducted in most parts of India[100].

Some studies in South India showed that *Fusarium spp* to be more common than *Aspergillus spp. Fusarium spp* have also been found to be the principal fungal pathogen in Florida, Paraguay, Singapore, Nigeria, Tanzania and Hong Kong. This phenomenon may be explained by difference in climate and the natural environment[23].

Jagadish Chandar *et al,* in 1993 studied that 8% fungal corneal ulcer were caused by *Acremonium spp* in *Chandigarh*.[41] Namrata Kumara *et al,* in 2002 reported mycotic keratitis in Patna documented 3.94% isolates as *Acremonium spp.*[71]

Verenkar M P *et al*, in 1998 study in Goa concluded 12.5% of corneal ulcer shows *Penicillium spp*[103]. Namrata kumara *et al*, in 2002 reported mycotic keratitis in Patna documented 7.89% isolates as of *Penicillium spp.*[71]

Willi's eye hospital, Philadelphia in May 2002 reported that *Candida albicans* was the most common agent in infectious keratitis[107].

DIFFERENCES BETWEEN BACTERIAL AND FUNGAL CORNEAL ULCERS

S. No.		Bacterial Ulcers	Fungal Ulcers
1.	History of Injury	Non-specific	With vegetable matter
2.	Onset after Injury	24-72 hours	One to two weeks
3.	Predisposing factors	Non-specific	Systematic immunosuppressives, local or systemic steroids therapy
4.	Course	Rapid	Usually slow but can be rapid if activated by steroids
5.	Clinical features	Proportionate	Signs out of proportion to symptoms.
6.	Description of ulcer	i. Moist look ii. Soft slough iii. Marked infiltration with gross destruction of tissue iv. Highly vascular v. Perforation common	• Dry necrotic look, yellowish-white in colour • Thick solid slough • Hyphate margins, satellite lesions, immune ring • Minimum vascularization • Perforation less common
7.	Hypopyon	Fluid and mobile	Dense and immobile

Liesegang and Foster in South Florida in 1999 studied in six hundred and sixty three patients (663), the fungal isolates contribute 20.1%, among the isolates *Fusarium spp* were the most common and *Aspergillus spp* was the next pathogen being isolated.[61]

Upadhyay *et al*, in Nepal reported that *Aspergillus spp* was to be predominant fungal pathogen and *Fusarium spp* were less commonly isolated[101].

Savithri Sharma *et al*, from Madurai studied that *Fusarium spp* showed high prevalence among the isolates[88].

ACANTHAMOEBA KERATITIS

Acanthamoeba keratitis has gained importance recently because of its increasing incidence, difficulty in diagnosis and unsatisfactory treatment.[3]

Etiology

Acanthamoeba keratitis is caused by Acanthamoebae, which are free living protozoans found in air, soil and fresh or brackish waters. They exist in both active (trophozoite) and dormant (cystic) forms.[97,87]

Predisposing Factors

1. *Acanthamoeba keratitis* may occur following a minor corneal abrasion.

2. Contact lens wearers who use distilled water and salt tablets instead of commercially prepared saline solutions for their lens care are at particular risk.

3. The fall of dust particles, trauma due to vegetable matter, contact with contaminated water etc have been found to be the predominant risk factors for *Acanthamoeba* keratitis.[3]

M.Jayahar Bharath *et al*,2009 reported that trauma due to vegetable matter was the major risk factor of *Acanthamoeba* keratitis.[7] The study on *Acanthamoeba keratitis* by S.Sharma *et al* 2000 was similar to the above study.[90]

Symptoms

Severe pain out of proportion to the degree of inflammation along with watering, photophobia, blepharospasm and blurred vision, is the characteristics features of *Acanthamoeba keratitis*[3]

Signs

Acanthamoeba keratitis evolves over several months as a gradual worsening keratitis with periods of temporary remissions.[3]

1. Initial lesions of *Acanthamoeba keratitis* are in the form of coarse and opaque streaks. Fine epithelial and subepithelial opacities are also seen.

2. Advanced cases show a central or paracentral ring-shaped lesion with stromal infiltrates. There is an overlying epithelial defect.

3. Severe cases show associated radial keratoneuritis, in the form of perineural infiltrates along corneal nerves.

Diagnosis

1. **Clinical Diagnosis:** It is difficult and is usually made by exclusion and with strong clinical suspicion in non-responsive patients being treated for viral, bacterial and fungal keratitis.
2. **Laboratory Diagnosis:** Staining of Corneal scrapings are helpful in identification of *Acanthamoeba cyst.*

a. Potassium hydroxide mount is reliable in experienced hands for recognition of *Acanthamoeba cysts.*

b. Calcofluor white stain is a chemifluorescent dye which stains the cysts of *Acanthamoeba* bright apple green.

c. Lactophenol cotton blue stained film is also useful for demonstration of *Acanthamoeba cysts* in corneal scrapings.

d. Culture on non-nutrient agar (*E.coli* enriched) showed trophozoites within 48 hours which gradually become cysts. E.coli prevents other organisms to grow whereas *Acanthamoeba* thrives on it.

3. **Confocal Microscopy:** *Acanthamoebae cysts* can be demonstrated in optically cut parallel sections of cornea under confocal microscopy.[3]

DIAGNOSTIC TECHNIQUES

Many fungal ulcers demonstrate no striking morphologic pattern and often it is not possible to differentiate clinically between fungal keratitis and bacterial keratitis[25].

To determine the causative organism meticulous collection of microbiological specimens is of critical importance. The corneal ulcer is scraped for microscopy, culture and for further investigations if indicated[74].

MICROSOPIC EVALUATION OF SMEARS

Gram stain

The smear is prepared from corneal scrapings and Direct Gram staining done to observe the bacteria and yeast like cell[51].

Bharathi *et al,* in 2006 studied 100% sensitivity of Gram stain procedure in the diagnosis of bacterial keratitis[6].

Feilmeier, Michael R *et al*, from Nepal in 2010 studied that smear microscopy is reliable in determining the etiology of the corneal infection and can be used to help guide initial therapy in this setting[26].

Noopur Gupta *et al,* in 2008 studied that smears prepared by corneal scraping and Gram staining done to observe the bacteria and yeast cells[74].

Giemsa stain is also useful to distinguish bacteria, fungi and *Acanthamoeba. Chlamydia* inclusion bodies can also be identified

with Giemsa stain[79].

10% Potassium Hydroxide Mount (KOH)

Corneal scrapings were placed on a glass slide with 10% KOH to see the fungal elements.[64]

In 1985 Araffa *et al*, concluded that KOH staining was as effective, much easier and less expensive than calcofluor white staining for detection of fungi in corneal tissue[2].

In 2007, Bharathi *et al*, reported that a potassium hydroxide smear is of greater diagnostic value in the diagnosis of fungal keratitis, *Nocardia* keratitis and *Acanthamoeba* keratitis[5].

1988, Sharma *et al*, reported that KOH preparation demonstrated fungus in 100 percent of total culture positive cases.[88]

Sharma *et al*, in 2000 concluded that 10% KOH mount could be used to demonstrate *Acanthamoeba* cysts in corneal scrapings there by permitting rapid presumptive diagnosis of *Acanthamoeba* Keratitis.[90]

Lactophenol cotton Blue mount (LPCB)

Corneal scraping are placed over a clean glass slide and a drop of lactophenol cotton blue stain is added over the specimen and a coverslip is placed taking care to avoid trapping of air bubbles. It was found to be effective for demonstration of fungal structures and *Acanthamoeba* cysts in corneal scrapings.[50]

Calcofluor white stain

Calcofluor white is a water soluble colourless textile dye and fluorescent whitener. It selectively binds to chitin and cellulose of the fungal cell wall. It fluoresces light blue when exposed to ultra violet light (346-365nm)[42].

The corneal scrapings are placed on a clean glass slide and 1 drop of 0.1% calcofluor white with 0.1% Evans blue and 1 drop 10% KOH are added. A coverslip is placed over the specimen and examined under fluorescent microscope. The morphology of smaller fungal elements was better appreciated in calcofluor white mount[92].

Acridine orange stain

Acridine orange dye has an affinity for nucleic acid. When fungi are stained with this dye, RNA component of the cell

fluoresces with shades of orange red and DNA component fluoresces green under fluorescent microscope.[42]

Acanthamoeba cysts fluoresces bright yellow to orange. This stain has been used for direct examination of corneal scrapings in cases of *Acanthamoeba* Keratitis[42].

Culture

Corneal scraping from patients with infectious keratitis due to trauma were inoculated on to Blood Agar Plate (BAP), Chocolate Agar Plate (CAP) for identify Bacterial etiology and to Sabouraud's Dextrose Agar (SDA) for fungal etiology and to Non - Nutrient agar (NN) media with lawn culture of *E.coli* for parasitic etiology.

Wihelmus *et al,* in 1994 studied that the culture media recommended for evaluation of suspected microbial keratitis have the potential to support the growth of the principal bacteria and fungi responsible for keratitis.[98],[107]

'O'Brien *et al,* in 1994 reported that the SDA agar should not contain cycloheximide which may inhibit the saprophytic fungi commonly responsible for ocular infection[76].

Slide culture technique

The slide culture is used to study morphology without disturbing, details particularly relationship between reproductive structure like conidia, conidiophores and hyphae. Fungal slide culture was performed in cases with doubtful morphology [55].

MOLECULAR DIAGNOSIS:

Polymerase Chain Reaction (PCR)

Sujith venayil *et al*, in 2009 studied that although PCR has several advantages due to its rapid and wide spread applicability to bacteria, fungi and viruses, the technique has various reported complexities and drawbacks as evidenced from their study also some of the limitations are logistic and some are technical[54,94].

TREATMENT OF BACTERIAL KERATITIS

Principles

1. **Control of Infection:** Infection is controlled by intensive local use of antibiotic drops. Broad spectrum antibiotic drops, ointment, are given frequently 4-6 times a day. Subconjunctival injections may also be given once or twice daily. Culture and sensitivity should be done before the application of antibiotics.

2. **Cleanliness:** Irrigation with warm saline or sodabicarb lotion is advised to wash away necrotic material, toxin, secretion and pathogenic organisms.
3. **Heat:** Heat prevents stasis and encourages repair of the ulcer. Hot fomentation may be given.
4. **Rest:** 1% atropine either as drops or ointment is applied 2-3 times a day. It paralyses the ciliary muscles and provides comfort to the eye by preventing ciliary spasm. There is associated iritis always in cases of corneal ulcer due to penetration of endotoxin across the endothelium in the anterior chamber. It also prevents most of the dangerous complications of iritis. Pad and bandage give rest to the eyeball by restricting its movements.
5. **Protection:** Pad and bandage protect the eye from dust, wind and harmful external agencies. A shield of dark glasses are used, if there is associated conjunctival discharge to avoid retention of secretion, which in turn favours bacterial growth due to warmth and stasis.

Topical administration is the method of treatment of choice, because it provides a rapid high level of drug in the cornea and anterior chamber.[96]

'O' Brien TP *et al*, in 1995 and Panda A *et al*, in 1991 concluded that initial regimens of fluroquinolone or aminoglycoside combined with a cephalosporin is effective in approximately 95% of cases of bacterial keratitis.[76,77]

Amikacin is a semi synthetic aminoglycoside that is useful in the treatment of infection due to gram negative infection resistant to gentamycin.[79]

In *Pseudomonas keratitis* ciprofloxacin is the drug of choice.[79]

TREATMENT OF FUNGAL KERATITIS

Principles

1. Scraping and debridement of the ulcer is useful in drug penetration.
2. 1% atropine eyedrops or ointment controls associated iritis and prevents synechiae formation.
3. Antifungal drugs – The available antifungal drugs are mainly fungistatic.

MEDICAL THERAPY

Antifungal drugs

The role of these drugs is limited due to few approved antifungal drugs and their poor penetration. Topical antifungals are to be instilled for a long-time, as the response is often delayed.[3]

ANTIFUNGAL THERAPEUTIC REGIMEN USED IN FUNGAL KERATITIS

1.	Amphotericin-B	Topical – 0.3% every hour. Taper over several weeks Subconjunctival 100-300mg on alternate day x1-2 doses Intravitreal 5-10ug Systemic – by infusion, 5-10mg total dose is given/day.
2.	Ketoconazole	Oral 200-400 mg/day for atleast 14 days
3.	Miconazole	Topical – 10mg/ml eyedrops every hour, taper gradually.
4.	Clotrimazole	Topical 1% eyedrop every hour. taper over several weeks
5.	Natamycin	Topical 5% drops every hour. taper over several weeks
6.	Flucytosine	Topical 10 mg/ml eye drop every hour, then taper gradually.
7.	Nystatin	Topical eye ointment is applied.

a. Topical

i. Natamycin (5%) eyedrops is instilled 1 hourly. It is effective against the most common fungi.

ii. Miconazole (1%) eye ointment is applied 5 times daily.

iii. Nystatin eye ointment is applied 5 times daily. It is only effective against *Candida* spp and is less potent.

iv. Topical Amphoterecin B (0.25%) is instilled 1 hourly and is effective against *Aspergillus spp* and *Candida spp*.

SYSTEMIC

Systemic antifungals are indicated, if the infection spreads to the sclera and there is impending perforation. e.g. oral ketoconazole or fluconazole 200 mg daily may be given for 2-3 weeks.

Cycloplegics such as atropine is used to prevent posterior synechiae formation and to control iritis by paralyzing the ciliary muscle. It also causes vasodilatation.[3]

Corticosteroids are contraindicated as they enhance fungal growth.

Thomas PA *et al*, in 2003 from India reported that Natamycin (5%) (or) Amphotericin B (.15%) remain the drug of choice for superficial keratitis.[99]

Pankaj K Agarwal *et al*, in 2001 from Calcutta concluded that Itraconazole is effective in treating mycotic corneal ulcers.[78]

Mohan *et al*, in 1989, reported success rate of 64.7%, when 1% Miconazole was used to treat smear positive keratitis.[68]

Newer agents such as triazoles (Posaconazole and Ravuconazole), Echinocandins, Sodarin derivates and the Nikkomycins might improve the treatment of fungal keratitis in future.[24,27]

TREATMENT OF ACANTHAMOEBA KERATITIS

Treatment of acanthamoeba keratitis is usually unsatisfactory, but the treatment mentioned below is usually followed.[3]

1. Non-specific treatment is on the general lines for corneal ulcer.
2. Specific medical treatment includes:

a. Topical treatment

1. Propamidine isethionate (Brolene) 0.1% drops are used hourly.
2. Neomycin drops
3. Polyhexamethylene biguanide (0.01-0.02%) drops are used hourly
4. Chlorhexidine drops
5. Paromomycin drops
6. 1% Clotrimazole drops
7. Polymyxin B drop

b. Systemic treatment

Oral ketoconazole 200mg may be given four times a day for 2-3 weeks.

c. Penetrating ketatoplasty is frequently required in non-responsive cases.

It is difficult to treat Acanthamoeba Keratitis. Chlorhexidine and Polyhexamethylene biguanide (PHMB) are the most effective drugs against trophozoites and cysts and are recommended as the first line therapy for Acanthamoeba keratitis. Medications have to be continued for 3-6 months after clinical resolution of infection to prevent relapses.[3]

AIM OF THE STUDY

AIM OF THE STUDY

- To isolate and identify the bacterial, fungal and parasitic aetiological agents in patient with infectious keratitis due to trauma.

- To analysis the correlation between aetiological agents of infectious keratitis due to trauma with the occupation of the patients.

- To evaluate the antibiotic susceptibility pattern of the bacterial isolates and study the beta lactamase production for their resistant isolates.

- To study the antibiotic susceptibility pattern of the fungal isolates by agar dilution and broth micro dilution method.

- To compare the antifungal susceptibility pattern for the fungal isolates by agar dilution and broth micro dilution method.

MATERIALS AND METHODS

MATERIALS AND METHODS

PERIOD OF STUDY

This is a cross sectional study undertaken over a period of one year from October 2011 to September 2012.

PLACE OF STUDY

This study was carried out at the Institute of Microbiology, Madras Medical College, Chennai and Regional Institute of Ophthalmology and Government General Hospital, Chennai.

STUDY GROUP

All patients presenting in the outpatient department with the history of trauma and signs and symptoms of infectious corneal ulcer such as pain, redness, watering of the eye, diminished vision and photophobia were included in the study.

ETHICAL CONSIDERATIONS

Written consent to participate in the study was obtained from the patients or their guardians after providing full

explanation of the study. This study was reviewed and approved by the Institutional Ethical Committee, Madras Medical College & Rajiv Gandhi Government General Hospital, Chennai-3. All data were handled confidentially and anonymously.

COLLECTION OF SPECIMENS:

Written consent from the participants (or) their guardians included in the study, was obtained after providing full explanation of the current study in their local language. All the data collected were kept confidential.

Specimens were collected from patients with infectious keratitis due to trauma, as per the proforma (Annexure) Informed consent from the patients and data were collected. Corneal scrapings were collected for the investigations by the Ophthalmologist.

Patient was made to lie down comfortably on a couch. The affected eye was cleansed with sterile normal saline using sterile swabs. Sterile 2% Xylocaine was applied to the eye, taking care not to apply too much, as it may inhibit the growth of the organism. Care was taken to see that the eyelids did not

contaminate the specimens. Eye speculum was used whenever necessary. No.15 and Bard Parker Blades were used to obtain scrapings from the ulcer.

The scraping were inoculated in a C-streak pattern on culture media (Blood Agar, Potato Dextrose Agar, Chocolate Agar and Non-nutrient Agar). The scraping is done by the Ophthalmologist under an operating microscope.

Direct Gram's stain and and 10% KOH wet mount were made on the direct scraping.

Incubate the Blood Agar and Chocolate Agar Plates at $37^{o}C$ in the presence of 5% CO_2 for 2-7 days.

Sabouraud's Dextrose Agar slant and Potato dextrose agar slant were incubated at $25^{0}C$ and $35^{0}C$ aerobically.

The culture plates and slants were observed for the growth of organisms every day / week. If bacterial growth was observed, staining (Gram's, Acid Fast, Modified Acid Fast) was performed.

Biochemical tests were done to identify the bacterial and fungal pathogens. Antibiotic susceptibility pattern was performed

to identify the susceptibility pattern of the isolates to the antibiotics.

If the fungal growth was observed, Lactophenol Cotton Blue (LPCB) staining was performed and the fungus was identified based on the spore morphology.

After 48-72 hours observe non - nutrient agar plate under low power and high power for the presence of Acanthomoeba cyst. The cyst present were further confirmed by calcofluor stain.

SPECIMEN PROCESSING

The following test were performed on the scrapings that were collected.

1. Gram Staining
2. Modified Acid Fast Staining
3. KOH Wet Mount
4. Giemsa Staining
5. Lactophenol Cotton Blue Staining(LPCB)
6. Slide culture method
7. Calcoflour staining

1. Gram Staining Procedure

Thin smear of the specimen was prepared on a clean and sterile glass slide. Then the smear was fixed by heating over a Bunsen burner flame. The smear was flooded with 1% crystal violet for 1 minute and washed with distilled water. The smear was flooded with Gram's iodine for 1 minute and washed with distilled water and decolorized with acetone, washed with distilled water and counter stained with dilute carbol fuschin for 30 seconds.

2. Modified Acid Fast Staining

Thin smear of the specimen was prepared and dried in the air. The smear was fixed by heating over a Bunsen burner flame. The smear was flooded with strong carbol fuschin stain for 5 minutes. Washed with distilled water and flooded with 1% sulphuric acid for 3 minutes. Washed with distilled water and counter stained with 3% methylene blue for 3 minutes. Washed with distilled water dried and examined under oil immersion objective of the microscope.

3. KOH Wet Mount

A clean glass slide was taken. The specimen was placed in

the centre of the slide. A drop of 10% KOH was added and a coverslip was placed over that and observed under the low and high power of the microscope.

4. Giemsa Staining

Air dried and fixed smear with absolute methanol for 2-3 minutes, was stained with Giemsa stain for 30 minutes, then the smear was allowed to air dry and observed under the oil immersion objectives of the microscope.

EXAMINATION OF INOCULATED MEDIA:

Sabouraud's Dextrose Agar slopes were observed periodically for growth at 25^{o}C and 35^{o}C and if it was inadequate, they were reincubated. The Sabouraud's dextrose agar slopes were examined daily during first week and twice a week for the next three weeks. Failure of growth even after six weeks was considered as negative for fungal growth and were discarded.

LACTOPHENOL COTTON BLUE STAIN:

The fungal growth was taken from Sabouraud's dextrose agar slope with spud and transferred onto the clean glass slide and two to three drops of Lactophenol cotton blue stain was added over the fungal growth. By using teasing needles the growth was

spread over the slide and coverslip was placed without trapping any air bubbles. The morphology of hyphae, conidia were observed under microscope and was correlated with macroscopic features.

RIDDLE'S SLIDE CULTURE METHOD:

This method was used to study the undisturbed morphological details of fungi particularly relationship between reproductive structures like conidia, conidiophores and hyphae. Fungal slide culture was performed in cases with doubtful morphology.

A round piece of filter paper was placed on the bottom of a sterile Petri dish. A pair of thin glass rods was placed on top of the filter paper to serve as support for the 3 inch x 1 inch glass microscope slide. 3 to 4 coverslips were placed within the Petridish and sterilized as a whole.

1x1 cm square block of Sabouraud's Dextrose Agar was cut from a Petridish by using sterile scalpel and transferred to the microscope slide.

Four sides of the agar block were inoculated with the fungal colony which is to be studied by using heavy 24 gauge nichrome wire. The agar block was covered with sterile coverslip in the Petridish. Moisten the filter paper with sterile water and place the lid on the Petridish.

The Petridish was incubated at room temperature and examined periodically for growth. When the growth appeared to be mature, the coverslip was gently lifted from the surface of the agar with a pair of forceps taking care not to disturb the mycelium adhering to the bottom of the coverslip.

The coverslip was placed on a small drop of Lactophenol cotton blue on a second glass slide. Likewise, the mycelium adhering to the surface of the original glass slide after the block was removed, also was stained with Lactophenol cotton blue stain and a fresh coverslip was overlaid.

The characteristic shape and arrangement of hyphae, conidia were observed microscopically.

The mycelia which adhere to the glass surface usually show characteristic microscopic appearance which may be lost if

needles are used to tease, as it happens in the routine Lactophenol cotton blue mounts. The slide culture may also be seen directly by placing under the low power of the microscope.

The cellophane tape preparation has come into greater use to overcome the obstacles of time consumption and requirement of extra equipment to prepare the slide culture. A piece of tape is gently laid over a portion of the fungal colony and slowly lifted to remove an area of the colony and placed on a microscope slide with a drop of Lactophenol cotton blue stain and examined under the low power objective of the microscope. This preparation becomes an instant slide culture, revealing relationship of the various fungal structures.

INTERPRETATION OF BACTERIAL CULTURE :

Bacterial isolates were identified by means of colony morphology, Gram staining, motility and biochemical reactions by standard microbiological techniques as recommended by Clinical and Laboratory Standards Institute (CLSI)

Interpretation of Fungal Culture

Inoculated SDA slopes were incubated at 25^0C and 35^0C for a minimum of six weeks before discarding as negative. These slants were inspected daily during the first week and twice weekly during the next three weeks for growth.

Identification of filamentous fungi was done by preparing Lacto Phenol Cotton Blue mount and studying the morphology of hyphae and conidial arrangement. In difficult, ambiguous cases where sporulation was inadequate, Riddle's slide culture technique was performed.

In case of yeasts, identification was done by Gram's stain morphology, germ tube test, morphology on corn meal agar, and biochemical tests by standard microbiological techniques as recommended by CLSI.

INTERPRETATION OF PARASITIC CULTURE

Corneal scrapings inoculated on to a non - nutrient agar plate for the cultivation of free living amoeba, where observed, after 48-72 hours observed under low power and high power

objective of the microscope for the presence of *Acanthaomoeba* cyst. The *Acanthaomoeba* cyst present were further confirmed by calcofluor stain.

Trophozoites of *Acanthamoeba* are 14-15 micrometer in size and actively motile at 37^{o}C and have numerous spiny *acanthopodia.* Cysts are smaller 10-25 mm, double walled, with wrinkled outer wall (ecto cyst) and a stellate polygonal inner wall (endo cyst)

ANTIMICROBIAL SUSCEPTIBLITY TESTING:

Antibiotic susceptibility testing was performed by the Kirby Bauer method on Mueller Hinton Agar (MHA) according to the CLSI protocols. The diameters of zones of inhibition were interpreted according to CLSI standards for each organism. Media and discs were tested for quality control using standard strains.

The following standard strains were used

1. *Staphylococcus aureus* - *ATCC 25923*
2. *Escherichia coli* - *ATCC 25922*
3. *Pseudomonas aeruginosa* - *ATCC 27853*

THE ANTIBIOTIC DISCS USED FOR BACTERIAL ISOLATES WERE:

Antimicrobial Agent	Inhibition zone in mm		
	Resistant	Intermediate	Sensitive
Oxacillin 1µg	10	11 – 12	13
Ciprofloxacin 5 µg	15	16 – 20	21
Amikacin 30 µg	14	15-16	17
Ceftazidime 30µg	14	15-17	18
Cefataxime 30µg	14	15-22	23
Gentamicin 10µg	12	13-14	15
Imipenem 10µg	13	14-15	16
Ofloxacin 5µg	12	13-15	16
Cotrimoxazole 1.25 µg /23.75µg	10	11-15	16
Chloramphenicol 30µg	12	13-17	18
Penicillin 10 units	28	-	29
Erythromycin 15µg	13	14-22	23

Detection of β lactamase enzyme production in gram negative bacilli:

A) DETECTION OF EXTENDED SPECTRUM β LACTAMASES (ESBL) PRODUCTION:

1. Isolates of gram negative bacilli showing the following resistance pattern were considered to be possible ESBL producing strains.

Antibiotic	Zone diameter for possible ESBL producing strain
Ceftazidime (30µg)	≤ 22mm
Cefataxime (30µg)	≤ 27mm
Ceftriaxone (30µg)	≤ 25mm
Aztreonam (30µg)	≤ 27mm

2. CLSI phenotypic confirmation method :

With a sterile bacterial loop 3-5 identical colonies were picked from a fresh overnight grown culture and inoculated into

5ml of nutrient broth. The broth was incubated at 35^0C for 2-4 hrs and the turbidity matched with 0.5 McFarland's standard. Lawn culture of the test organism was made on to MHA plate. Antibiotic discs like Ceftazidime (CAZ 30µg) and Ceftazidime / Clavulanic acid (CAZ / CA / 30µg / 10 µg) (Himedia, Mumbai) were placed onto the plate and incubated at 35^0C overnight. A ≥ 5mm increase in zone diameter for Ceftazidime tested in combination with Clavulanic acid than its zone when tested alone confirmed an ESBL producing organism.

DETECTION OF METHICILLIN RESISTANCE IN *STAPHYLOCOCCUS AUREUS :*

1. **Disc Diffusion Method**

Colonies isolated from agar culture plate were suspended directly into broth, vortexed to reach 0.5 McFarlands standard. A lawn culture of the Staphylococcal colonies was made on the MHA plate and Oxacillin or Cefoxitin disc were applied. Incubation was at 35^0C for 24 hours in ambient air. According to CLSI criteria with 1µg Oxacillin disc, diameters of ≤ 10, 11-12, ≥ 13mm corresponded to categorization as resistant, intermediate or susceptible. With 30µg Cefoxitin disc diameter

of ≤ 19 or ≥ 20mm corresponded to resistant or susceptible to Oxacillin.

2. Minimum Inhibitory Concentratin (MIC) for detecting Vancomycin resistance :

1. Culture media cation adjusted Mueller Hinton Broth (pH 7.2-7.4)

2. Preparation of stock antibiotic solution :

Antibiotic stock solution can be prepared using the formula

$$= \frac{1000}{P} \times V \times C = w$$

Where p= potency of the antibiotic in relation to the base. (For vancomycin, p=950/1000 mg : Himedia)

V= Volume of the stock solution to be prepared (10 ml)

C = final concentration of the antibiotic solution (1024 µg /ml).

W=weight of the antibiotic to be dissolved in the volume

3. **Scheme for preparing dilution of antibiotics**

Arrange two rows of test tubes in the rack (1 row for the test & 2nd for ATCC control). Using sterile syringe transfer 2ml of MH broth to the uricol container containing the working stock solution

(128µg/ml concentration) From this transfer 1 ml to the first tube in each row. Now we have 2ml of the diluted antibiotic in the uricol container. Using sterile syringe add 2ml of MH broth to the 2ml to the second tube in each row. Repeat this procedure till the 8^{th} tube. Place 1 ml of the antibiotic free broth in the last tube in each row (growth control). The sterility controls for the antibiotic solution is kept.

4. **Inoculum preparation for the test and ATCC control and incubation**

Take 9.9ml of MH broth in a uricol container. Add 0.1 ml of 0.5 MCFarland turbidity matched test organism broth. Mix well, transfer 1 ml of inoculum using 2 ml syringe to each tube containing antibiotic dilutions and also to the control tube. Similarly, repeat the procedure for ATCC control strain. Incubate the rack at 37^0C for overnight. Observe the MIC of ATCC control strain. If it is out of the sensitive range, the test is invalid. Read for the test organism. The lowest concentration of the antibiotic in which there is no visible growth will be the MIC for the drug & for the test organism.

ANTI FUNGAL SUSCEPTIBILITY TESTS

The antifungal susceptibility testing testing was done by two methods

1.Disc diffusion method

2.Agar dilution method

3.Broth microdilution method

The Clinical Laboratory Standards Institute (CLSI) subcommittee on Antifungal susceptibility Tests has developed a reproducible procedure for antifungal susceptibility testing of filamentous fungi by a broth microdilution. Recently an agar dilution method has been developed for testing filamentous fungi by diffusion methodology.

Inoculum preparation :

All organisms were subcultured onto Potato dextrose agar, incubated at 35^0C for 7 days. The culture was covered with 1 ml of sterile 0.85% saline and a suspension prepared by gently probing the colonies. Addition of 1 drop of Tween 20 will help dispersion of Aspergillus conidia. The resulting mixture of conidia

and hyphal elements was withdrawn and transferred to a sterile tube and allowed to settle. The uniform suspension was transferred to a screw capped tube and vortexed. The densities of the conidia or the sproangiospore suspensions were read and adjusted to a optical density of 0.09-0.11 for *Aspergillus spp*.

Disc Diffusion Method :

Disc Diffusion test was performed on Mueller-Hinton agar plates supplemented with 2% Glucose and 0.5µg/ml of Methylene Blue.

The entire dried agar surface was evenly streaked in three different directions with a sterile cotton swab dipped into the inoculums suspension. The plate was allowed to dry for 20 minutes. Using a pair of flame sterilized forceps the antifungal discs were applied onto the surface of the inoculated plate. The plates were incubated at 35^{o}C for 48 hours. The plates were read at 24 hrs and 48 hrs.

The following commercial HI-Media antifungal disks were used.

Amphotericin B	100 units	Itraconazole	10µg
Fluconazole	10 µg	Nystatin	10 µg

The following standard strains were tested each time to ensure quality control.

Aspergillus flavus	ATCC 204304
Aspergillus fumigates	ATCC 204305

INTERPRETATION:

Zone diameters were measured at the point where there was prominent reduction of growth. The results were compared with broth microdilution method for respective fungal isolates.

AGAR DILUTION METHOD PROCEDURE

- 1.8 ml of nutrient agar poured in the test tube and allowed to cool at 50^{o}c.
- From the stock solution, 0.2ml of drug dilution added in the descending concentration to NA slope
- 100 µl of standardised inoculums added.

- All the test tubes incubated 30^0c for 2 days.
- Examining macroscopically for growth
- Lowest concentration of the drug which permitted no growth after 2-3 day is taken as MIC.

BROTH MICRODILUTION METHOD:

Amphotericin B and Itraconazole powders and voriconazole powders were obtained form HiMedia, Mumbai and Pharma Fabricon respectively. Their essay potency were 750 µg/mg each.

$$\text{Weight (mg)} = \frac{\text{volume (ml)x desired concentration (µg/ml)}}{\text{Assay potency (µg/ml)}}$$

$$\text{Volume (ml)} = \frac{\text{weight (mg) x assay potency (µg/ml)}}{\text{Concentration (µg/ml)}}$$

STOCK SOLUTION

Solvent used is Dimethyl sulfoxide (DMSO) for Amphotericin B and Itraconazole and voriconazole. Stock solution of 1600 (µg/ml) is prepared. A series of dilutions at 100 times the

final concentration was prepared from the antifungal stock solution in the same solvent. Each intermediate solution was then further diluted to final strength in the test medium. This procedure was done to avoid dilution artifacts that result from precipitation of compounds with low solubility in aqueous media.

Media : RPMI 1640 (with glutamine, without bicarbonate and phenol red as pH indicator) Hi-Media, Mumbai.

INCUBATION :

All microtitre plates were incubated at 35^0C and examined for MIC determination at 48 hrs.

INTERPRETATION OF MIC :

By visual examination, MIC was defined as the lowest drug concentration that showed 100% growth inhibition compared to the growth control well.

The test was read when the growth control shows adequate growth, which is typically 24-48 hours for most moulds, but it could be up to 96 hours. Read MICs the first day that the growths

controls showed the visible growth and then 24 hours later.

Scores were given as follows, (1) 0-optically clear (2) 1 + = slightly hazy (3) 2+ prominent reduction in turbidity compared with that of the drug-free growth control. 3+ = slight reduction in turbidity compared with that of the drug-free growth control. 4+= no reduction in turbidity compared with that of the drug-free growth control.

STATISTICAL ANALYSIS :

A Statistical analysis was carried out using statistical package for social science (SPCS) and Epi-info software in a statistician. The proportional date of the cross sectional study was tested using pearson's chi-square analysis and binomial proportion test.

RESULTS

TABLE OF RESULTS

TABLE 1: CULTURE POSITIVITY IN INFECTIOUS KERATITIS DUE TO TRAUMA (n=150)

Total No.of Samples	No.of Culture Positive Samples	Percentage of culture Positivity	Total No. of Bacterial agent	Total No. of Fungal agent	Total No. of Parasitic agent	Total No. of Mixed growth
150	94	62.66%	31	54	6	3

Out of total 150 samples, 94 aetiological agents were isolated, samples leading to 62.66% culture positivity. Among Bacterial fungal Parasitic agents isolated fungi was the predominant aetiological in patient with infectious keratitis due to trauma.

TABLE 2: GENDER DISTRIBUTION OF INFECTIOUS KERATITIS DUE TO TRAUMA (n=150)

Gender	Total No.of Cases	No. of Culture Positive	Percentage
Male	90	64	71.11%
Female	60	30	50.00%

Male gender have increased incidence of keratitis, probably because of their occupation. This was found to be statistically significant p value =0.009 Significant.

FIGURE - 1

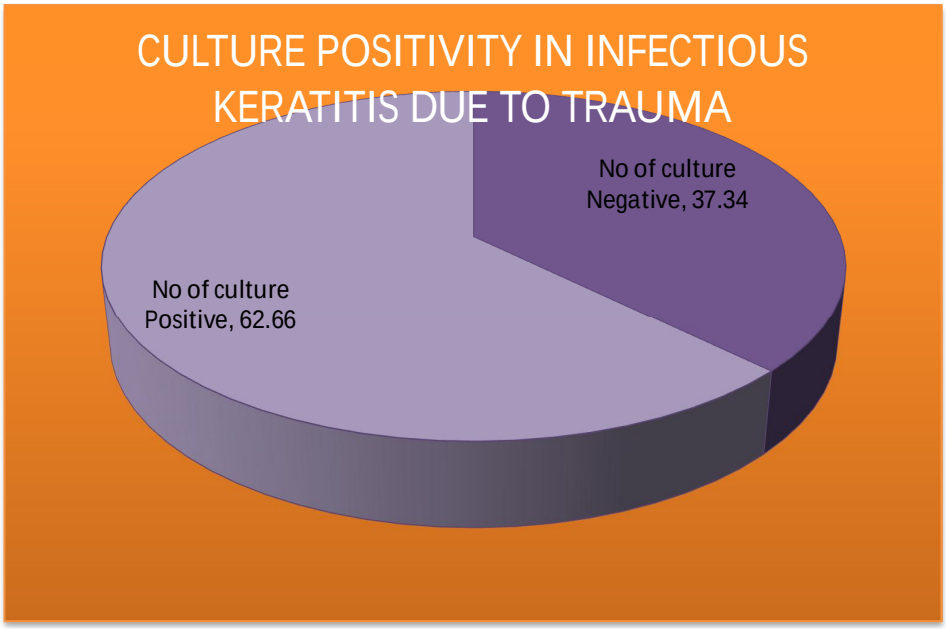


FIGURE - 2

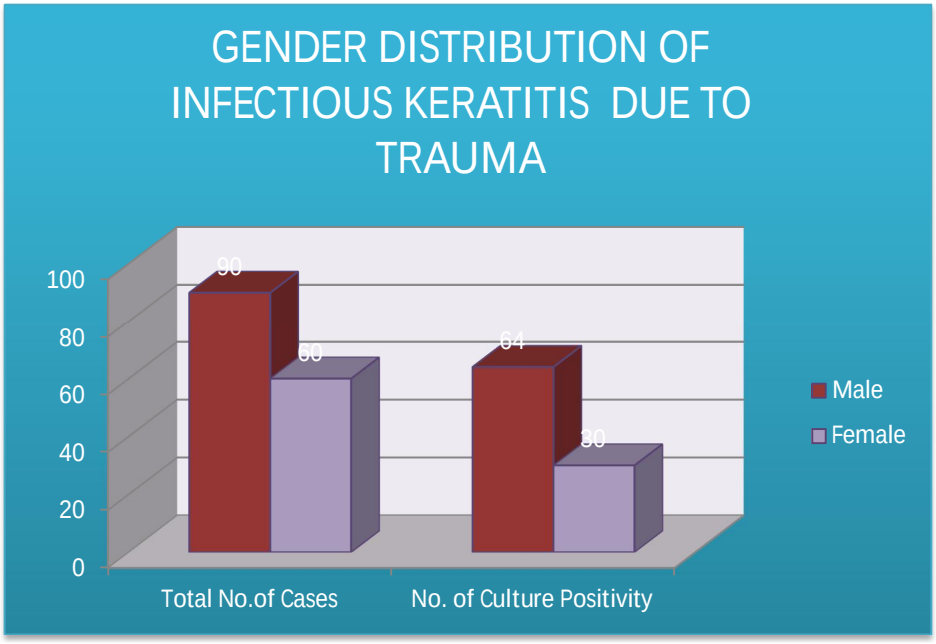


TABLE 3: AGEWISE DISTRIBUTION OF INFECTIOUS KERATITIS DUE TO TRAUMA (n=150)

Age in years	Total No.of Cases	No. of Culture Positive	Percentage
<10	2	1	1.06%
11-20	20	11	11.70%
21-30	21	8	8.51%
31-40	18	15	15.95%
41-50	22	14	14.89%
51-60	39	27	28.72%
61-70	20	16	17.02%
>71	8	2	2.12%
Total	**150**	**94**	**100%**

Prevalence of infectious keratitis due to trauma was more common in 51-60 yrs age group (28.72%)

TABLE 4: DISTRIBUTION OF INFECTIOUS KERATITIS DUE TO TRAUMA IN RURAL AND URBAN AREAS (n=150)

Total	Rural	Urban
150	97	53
Percentage	64.66%	35.34%

Incidence of infectious keratitis due to trauma was more in rural population compared to urban population which was statistically significant (64.66%). p value =0.005.

FIGURE - 3

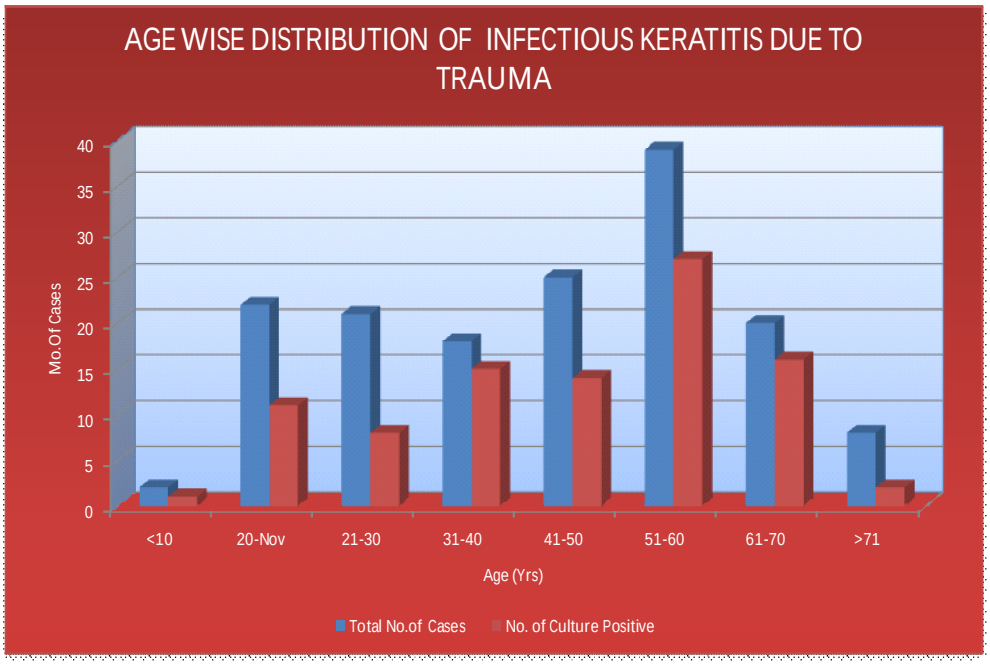


FIGURE - 4

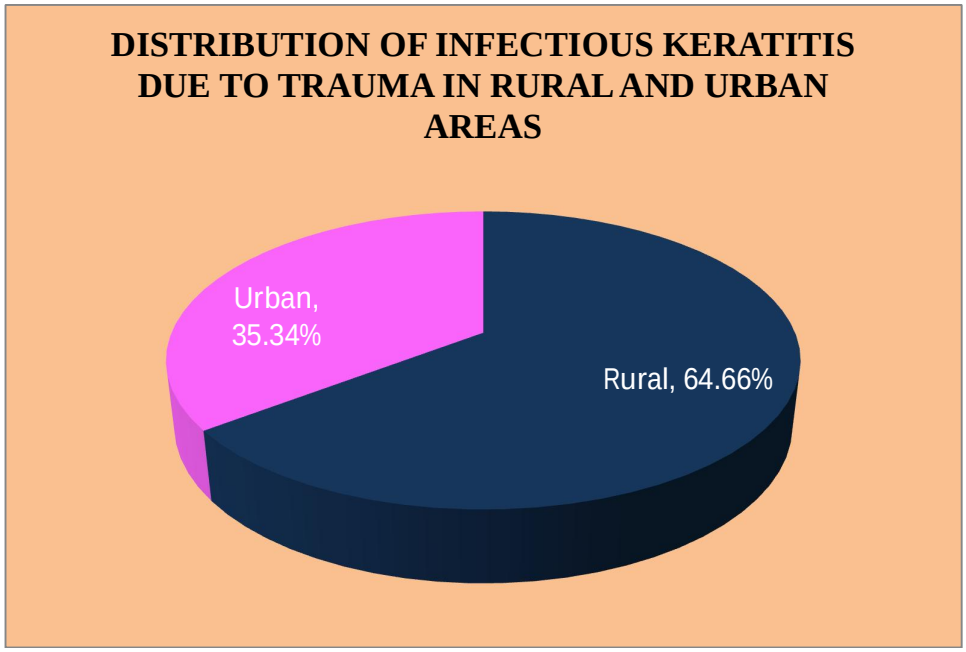


TABLE 5: GENDER DISTRIBUTION OF AETIOLOGICAL AGENTS

Group	Agent	Male	Female	Grand Total
Fungal Agents	*Aspergillus flavus*	8	6	14
	Aspergillus niger	7	4	11
	Aspergillus fumigatus	5	3	8
	Fusarium spp	9	3	12
	Curvularia spp	1	1	2
	Penicillium spp	2	1	3
	Graphium spp	2	-	2
	Scopulariopsis spp	1	1	2
Samples with pure fungal growth		**35**	**19**	**54 (57.44%)**
Bacterial Agents	*Staphylococcus. aureus*	5	2	7
	Staphylococcus. epidermidis	4	2	6
	Pseudomonas aeruginosa	3	2	5
	Klebsiella Pneumoniae	3	2	5
	Acinetobacter boumannii	2	2	4
	Escherichia coli	2	2	4
Sample with pure bacterial growth		**19**	**12**	**31 (32.97%)**
Mixed growth	*Pseuodmonas aeruginosa + A.fumigatus*	2	0	2
	Staph. aureus + A.niger	0	1	1
Samples with mixed Microbial Growth		**2**	**1**	**3 (3.19%)**
Parasitic Agent	*Acanthamoeba spp*	4	2	6
Sample with Pure parasitic growth		**4**	**2**	**6, 6.38%**
Total culture positive sample		**60**	**34**	**94, 100%**

FIGURE - 5

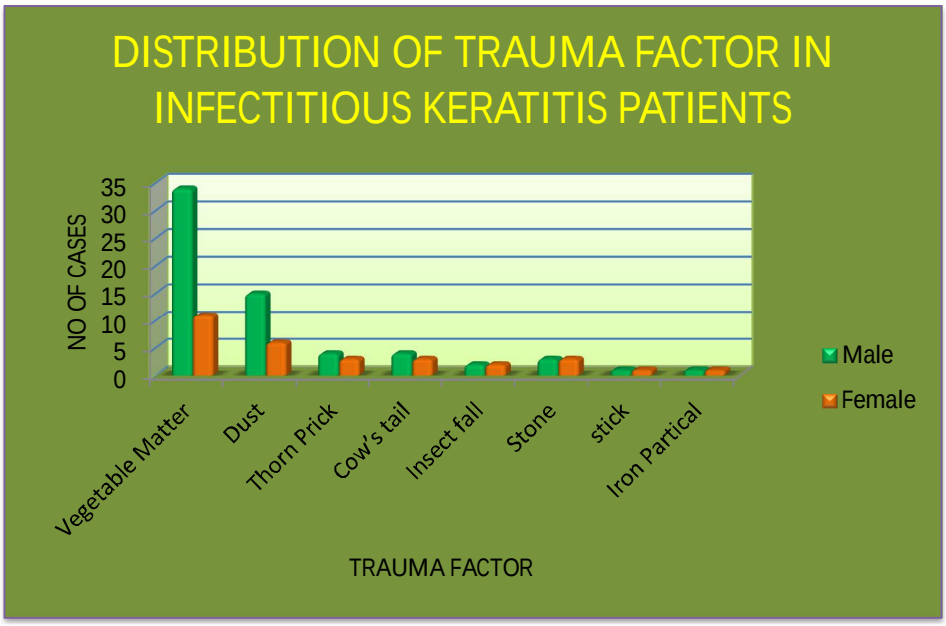


FIGURE - 6

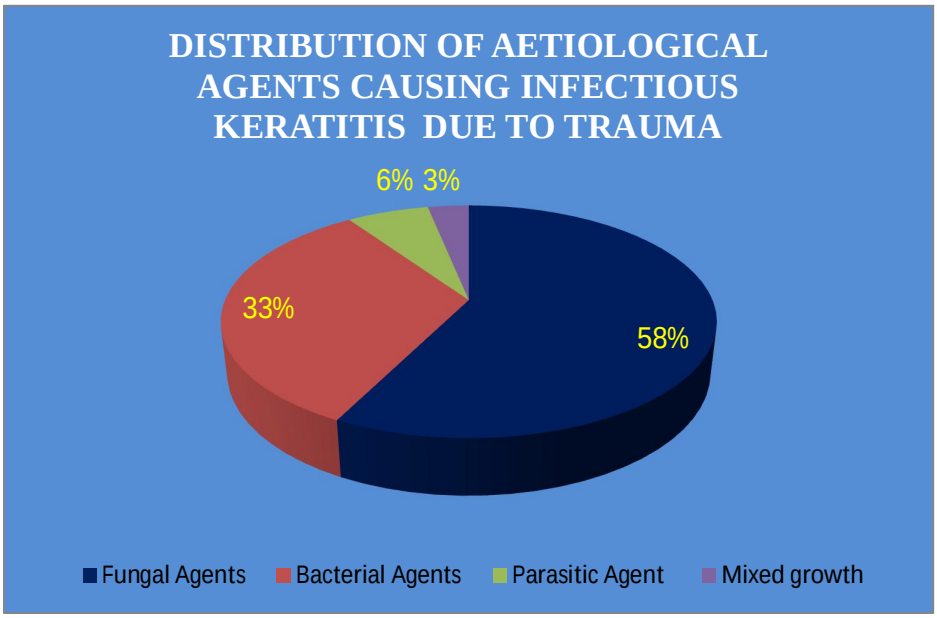


TABLE 6: DISTRIBUTION OF TRAUMA FACTORS IN INFECTIOUS KERATITIS DUE TO TRAUMA

Trauma Factor	Culture +ve Cases			Percentage
	Male	Female	Total	
Vegetable Matter	34	11	45	47.87%
Dust	15	6	21	22.34%
Thorn Prick	4	3	7	7.44%
Cow's tail	4	3	7	7.44%
Stone	3	3	6	6.38%
Insect fall	2	2	4	4.25%
stick	1	1	2	2.12%
Iron Partical	1	1	2	2.12%
Total	**64**	**30**	**94**	**100%**

In majority of the traumatic infectious keratitis patients, vegetable matter was the commonest traumatic factor which contributed to 47.87%.

FIGURE - 7

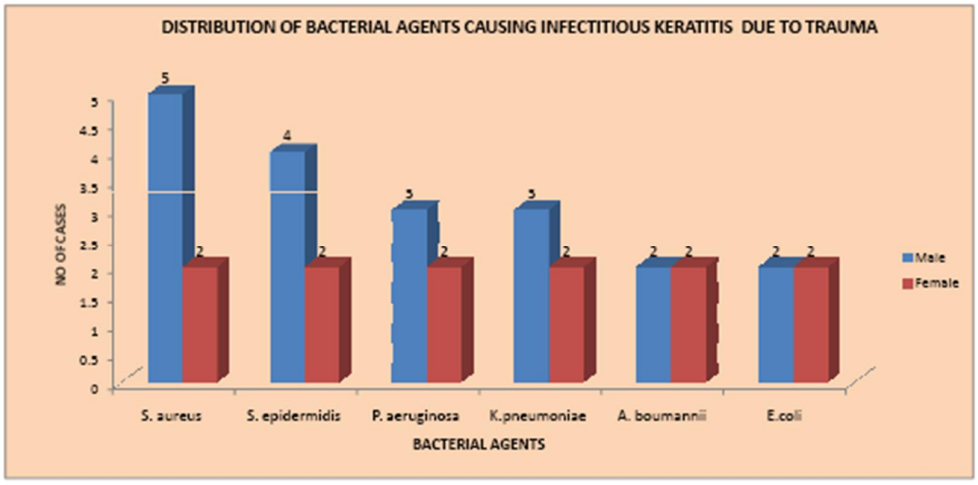


FIGURE - 8

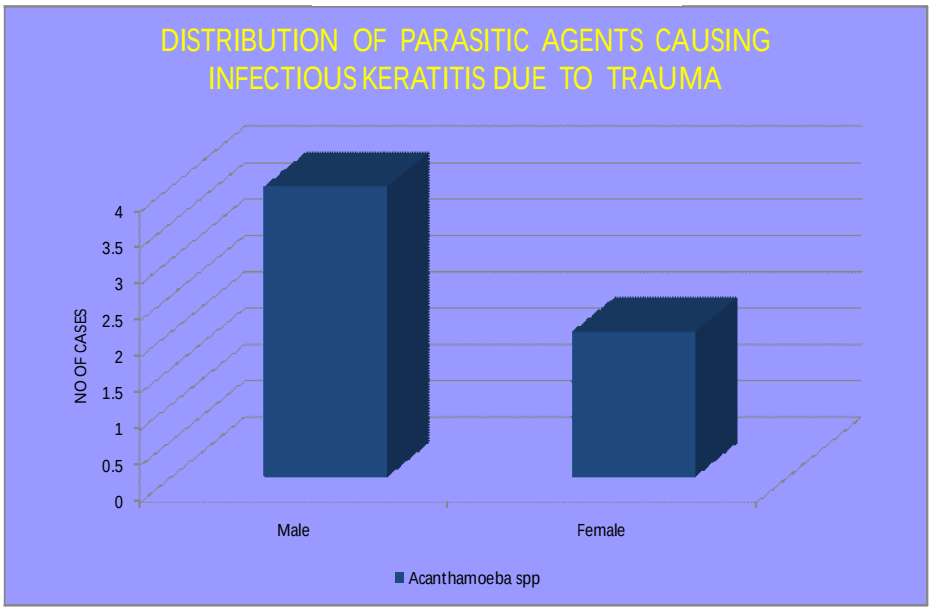


TABLE 7: DISTRIBUTION OF BACTERIAL AGENT CAUSING INFECTIOUS KERATITIS DUE TO TRAUMA

Bacterial Agent	Total No. of Isolates	Percentage
Staphylococcus. aureus	9	26.47%
Staphylococcus. epidermidis	6	17.64%
Pseudomonas aeruginosa	6	17.64%
Klebsiella pneumoniae	5	14.70%
Acinetobacter boumannii	4	11.76%
Escherichia coli	4	11.76%
Total	**34**	**100%**

Staphylococcus aureus was the most common bacterial agent (26.47%) causing infectious keratitis due to trauma.

TABLE 8: DISTRIBUTION OF FUNGAL AGENT CAUSING INFECTIOUS KERATITIS DUE TO TRAUMA

Fungal Agent	Total No. of Isolates	Percentage
Aspergillus flavus	14	24.86%
Aspergillus niger	12	21.05%
Aspergillus fumigatus	10	17.52%
Fusarium spp	12	21.05%
Curvularia spp	2	3.50%
Penicillium spp	3	5.26%
Graphium spp	2	3.50%
Scopulariopsis spp	2	3.50%
Total	**57**	**100%**

Aspergillus spp was the most common fungal agent (63.15%) causing infectious keratitis due to trauma.

FIGURE - 9

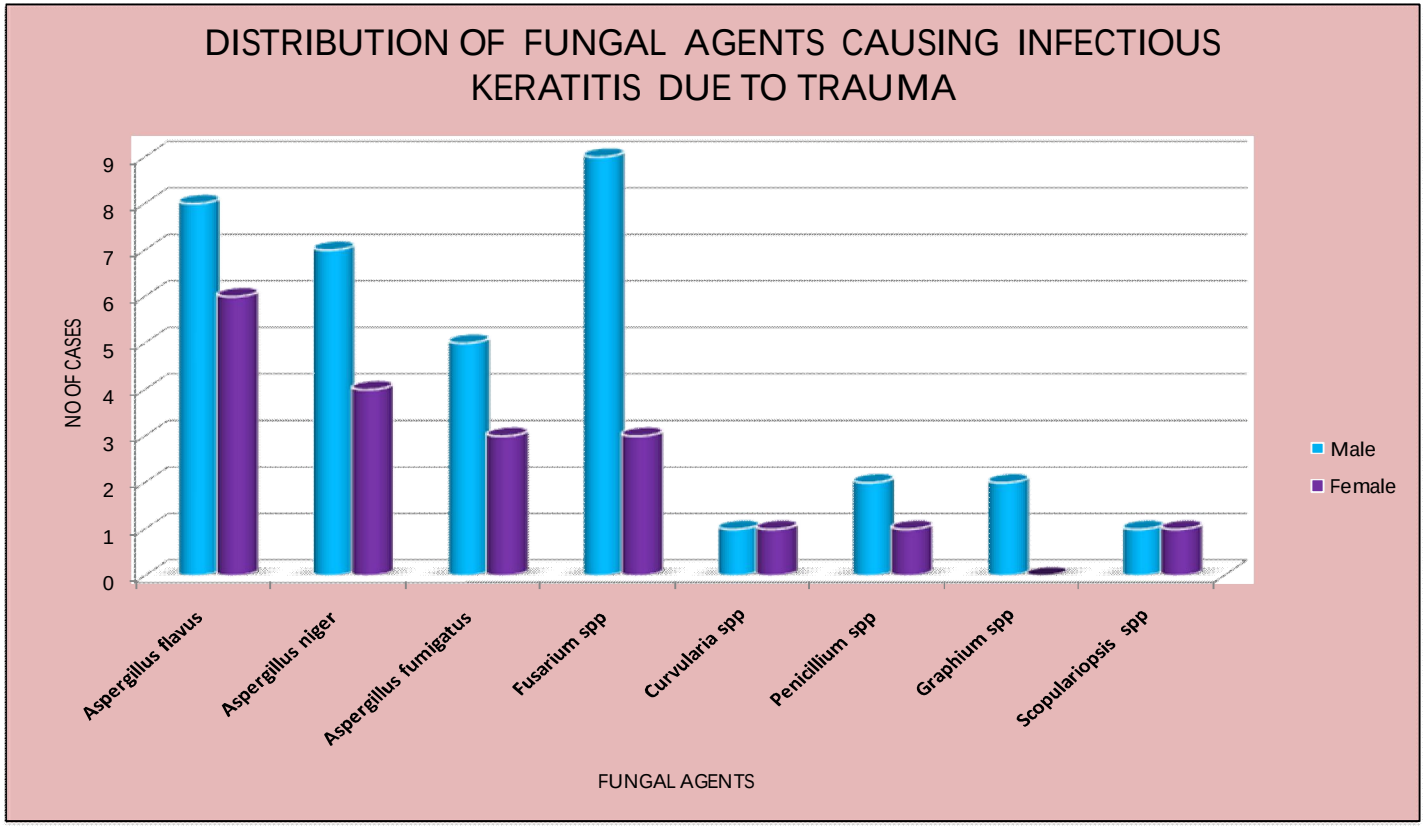

DISTRIBUTION OF FUNGAL AGENTS CAUSING INFECTIOUS KERATITIS DUE TO TRAUMA
NO OF CASES
0
1
2
3
4
5
6
7
8
9
Aspergillus flavus
Aspergillus niger
Aspergillus fumigatus
Fusarium spp
Curvularia spp
Penicillium spp
Graphium spp
Scopulariopsis spp
FUNGAL AGENTS
Male
Female

TABLE 9: DISTRIBUTION OF PARASITIC AGENT CAUSING INFECTIOUS KERATITIS DUE TO TRAUMA

Parasitic agent	Total No. of Isolates	Percentage
Acanthamoeba spp	6	6.38%

Acanthamoeba spp was the only parasitic agent isolated in infectious keratitis cases due to trauma (6.38%)

TABLE 10: ANTIBIOTIC SUSCEPTIBILITY PATTERN OF BACTERIAL ISOLATES IN INFECTIOUS KERATITIS DUE TO TRAUMA

Organism	Total No.	Amikacin		Gentamicin		Ciprofloxacin		Ofloxacin		Cefataxime		Cotrimoxazole		Cephalexin		Vancomycin		Oxacillin	
Staphylococcus. aureus	9	9	100%	-	-	7	77%	7	77%	8	88%	5	55.5%	7	77%	9	100%	7	77%
Staphylococcus. epidermidis	6	6	100%	-	-	6	100%	-	-	6	100%	4	66%	4	66%	6	100%	4	66%
Pseudomonas aeruginosa	6	6	100%	5	83%	4	66%	5	83%	4	66%	-	-	5	83%	-	-	-	-
Klebsiella pneumoniae	5	5	100%	4	80%	5	100%	4	80%	3	60%	-	-	4	80%	-	-	-	-
Acinetobacter boumanii	4	3	75%	3	75%	2	50%	2	50%	3	75%	-	-	3	75%	-	-	-	-
Escherichia coli	4	3	75%	3	75%	4	100%	3	75%	3	75%	-	-	3	75%	-	-	-	-
Percentage of Sensitivity	**34**		**94.11%**		**44.11%**		**82.35%**		**61.76%**		**79.41%**		**26.47%**		**76.47%**		**100%**		**32.35%**

Most of the bacterial isolates were susceptible to Amikacin, (94.11%) Two isolates of *S.aureus* were found to be methicillin resistant *Staphylococcus aureus* (MRSA) and among the *Staphylococcus epidermidis* two were found to be methicillin resistant.

FIGURE - 10

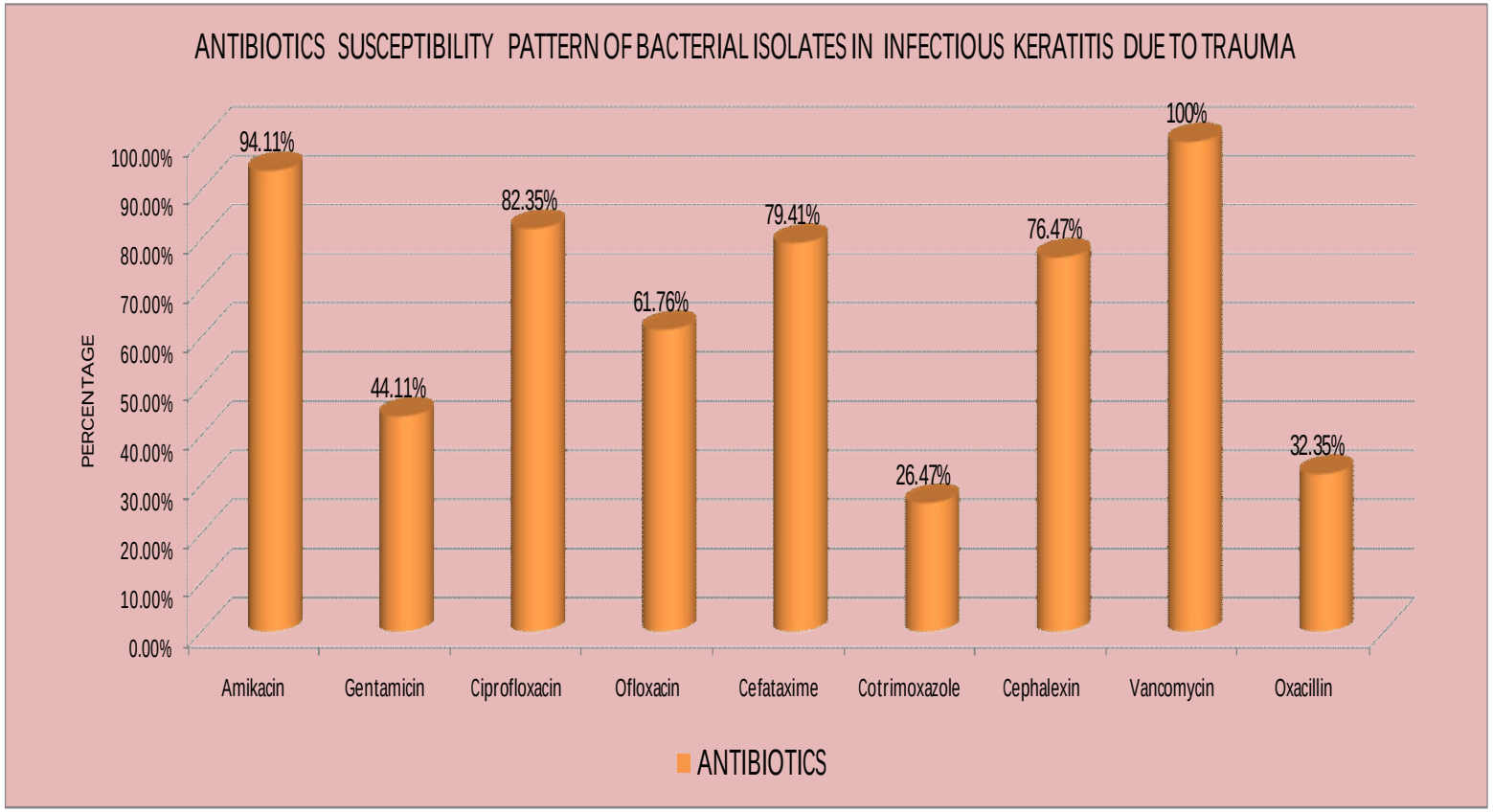

ANTIBIOTICS SUSCEPTIBILITY PATTERN OF BACTERIAL ISOLATES IN INFECTIOUS KERATITIS DUE TO TRAUMA
PERCENTAGE
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
94.11%
44.11%
82.35%
61.76%
79.41%
26.47%
76.47%
100%
32.35%
Amikacin
Gentamicin
Ciprofloxacin
Ofloxacin
Cefataxime
Cotrimoxazole
Cephalexin
Vancomycin
Oxacillin
ANTIBIOTICS

TABLE 11: MIC OF VANCOMYCIN FOR STAPHYLOCOCCUS AUREUS

Organism	Minimum inhibitory concentration break- point			
	0.25 μg/ml	0.5 μg/ml	1 μg/ml	>2 μg/ml
Staph.auerus (2)	1	1	-	-

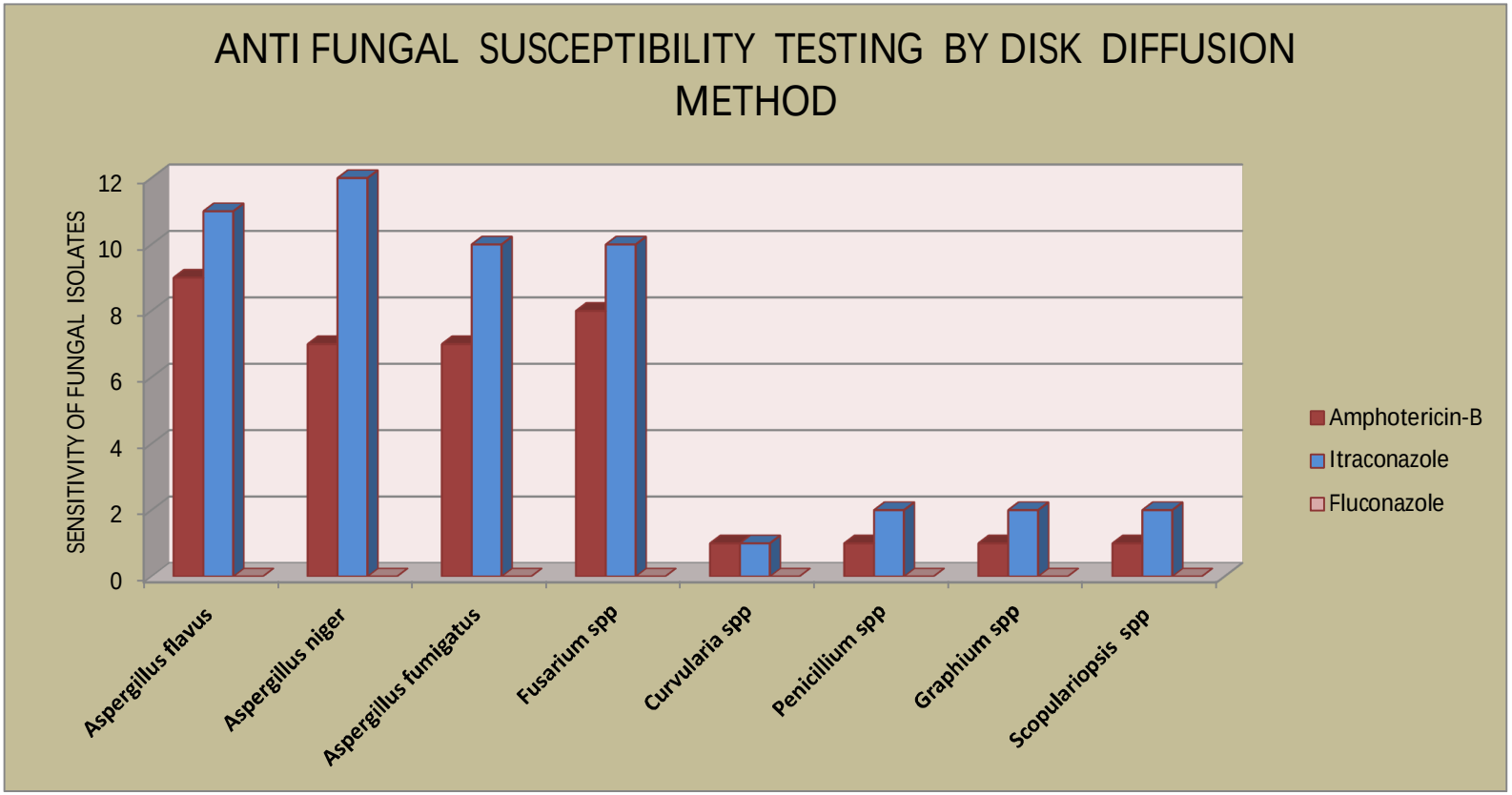
MIC for the MRSA isolates were studied and found to be 100% sensitive to vancomycin. The break point concentration were 0.25 μg/ml and 0.5μg/ml for the isolates.

TABLE 12: ANTIFUNGAL SUSCEPTIBILITY PATTERN BY DISC DIFFUSION TEST METHOD

Agent	No.of isolates	Amphotericin-B (20μg) S>15 mm	Itraconazole (10μg) S>23 mm	Fluconazole (10μg) S>19 mm
Aspergillus flavus	14	9 (64%)	11 (78%)	R
Aspergillus niger	12	7 (58%)	12(100%)	R
Aspergillus fumigatus	10	7 (70%)	10 (100%)	R
Fusarium spp	12	8 (66%)	10 (83%)	R
Curvularia spp	2	1 (50%)	1 (50%)	R
Penicillium spp	3	1 (33%)	2 (66%)	R
Graphium spp	2	1 (50%)	2 (100%)	R
Scopulariopsis spp	2	1 (50%)	2 (100%)	R

R- Resistant

FIGURE - 11


ANTI FUNGAL SUSCEPTIBILITY TESTING BY DISK DIFFUSION METHOD
SENSITIVITY OF FUNGAL ISOLATES
0
2
4
6
8
10
12
Aspergillus flavus
Aspergillus niger
Aspergillus fumigatus
Fusarium spp
Curvularia spp
Penicillium spp
Graphium spp
Scopulariopsis spp
Amphotericin-B
Itraconazole
Fluconazole

Itraconzole was found to be more effective against Aspergillus spp. All fungal isolates were resistant to fluconazole (<15 mm)

TABLE 13: MINIMUM INHIBITORY CONCENTRATION FOR AMPHOTERICIN B - AGAR DILUTION METHOD

Organism	0.25 µg	0.5 µg	1 µg	2 µg	4 µg	8 µg	16 µg	32 µg	64 µg
Aspergillus Flavus	3	2	5	2	1	-	1	-	-
Aspergillus niger	2	3	3	1	1	1	1	-	-
Aspergillus Fumigatus	1	3	1	2	1	1	-	1	-
Fusarium spp	3	1	3	3	1	1	-	-	-
Curvularia spp	-	1	1	-	-	-	-	-	-
Penicillium spp	-	1	1	-	1	-	-	-	-
Graphium spp	-	1	1	-	-	-	-	-	-
Scopulariopsis spp	-	-	1	1	-	-	-	-	-

28/36 (84.84%) of *Aspergillus Spp*, 10/12 (83.83%) of *Fusarium Spp*, 100% of *Curvularia Spp*, *Graphium Spp* and *Scopulariopsis spp* 2/3 (66.66%) of *Penicillium spp* showed sensitive range for Ampotericin B (MIC < 2 µg/ml).

TABLE 14: MINIMUM INHIBITORY CONCENTRATION FOR ITRACONAZOLE - AGAR DILUTION METHOD

Organism	0.25 µg	0.5 µg	1 µg	2 µg	4 µg	8 µg	16 µg	32 µg	64 µg
Aspergillus Flavus	3	4	2	4	1	-	-	-	-
Aspergillus niger	2	3	2	3	1	1	-	-	-
Aspergillus Fumigatus	-	2	3	2	1	-	-	1	1
Fusarium spp	2	3	2	3	1	1	-	-	-
Curvularia spp	1	-	1	-	-	-	-	-	-
Penicillium spp	1	-	1	1	-	-	-	-	-
Graphium spp	-	1	1	-	-	-	-	-	-
Scopulariopsis spp	-	-	1	1	-	-	-	-	-

Itraconzole was found to be more sensitive MIC≤2µg/ml against most of the fungal isolates out Of 57 fungal isolates 49 isolate had MIC≤2µg/ml.

TABLE 15: MINIMUM INHIBITORY CONCENTRATION FOR VORICONAZOLE - AGAR DILUTION METHOD

Organism	0.25 µg	0.5 µg	1 µg	2 µg	4 µg	8 µg	16 µg	32 µg	64 µg
Aspergillus Flavus	4	3	4	2	1	-	-	-	-
Aspergillus niger	3	2	4	3	-	-	-	-	-
Aspergillus Fumigatus	1	3	4	2	-	-	-	-	-
Fusarium spp	4	3	3	1	1	-	-	-	-
Curvularia spp	-	1	1	-	-	-	-	-	-
Penicillium spp	1	-	1	1	-	-	-	-	-
Graphium spp	-	1	1	-	-	-	-	-	-
Scopulariopsis spp	-	-	1	1	-	-	-	-	-

Voriconzole was found to be more sensitive MIC<2µg/ml against fungal isolates out of 57 fungal isolates 55 isolate had MIC≤2µg/ml.

TABLE 16: MINIMUM INHIBITORY CONCENTRATION FOR AMPHOTERICIN B - BROTH MICRODILUTION METHOD

Organism	0.25 µg	0.5 µg	1 µg	2 µg	4 µg	8 µg	16 µg	32 µg	64 µg
Aspergillus Flavus	2	3	4	2	1	1	1	-	-
Aspergillus niger	1	2	4	2	1	1	1	-	-
Aspergillus Fumigatus	1	2	3	1	1	-	-	1	1
Fusarium spp	3	2	3	3	1	-	-	-	-
Curvularia spp	-	-	1	1	-	-	-	-	-
Penicillium spp	-	1	1	1	-	-	-	-	-
Graphium spp	-	-	1	1	-	-	-	-	-
Scopulariopsis spp	-	1	1	-	-	-	-	-	-

Amphotericin B was found to be more sensitive MIC<2µg/ml against fungal isolates out of 57 fungal isolates 47 isolate had MIC≤2µg/ml.

TABLE 17: MINIMUM INHIBITORY CONCENTRATION FOR ITRACONAZOLE - BROTH MICRODILUTION METHOD

Organism	0.25 µg	0.5 µg	1 µg	2 µg	4 µg	8 µg	16 µg	32 µg	64 µg
Aspergillus Flavus	2	3	4	4	1	-	-	-	-
Aspergillus niger	2	3	2	3	1	1	-	-	-
Aspergillus Fumigatus	1	2	3	2	-	-	-	1	1
Fusarium spp	2	1	3	5	1	-	-	-	-
Curvularia spp	-	-	1	1	-	-	-	-	-
Penicillium spp	-	1	1	1	-	-	-	-	-
Graphium spp	-	-	1	1	-	-	-	-	-
Scopulariopsis spp	-	1	1	-	-	-	-	-	-

Ireaconazole was found to be more sensitive MIC<2µg/ml against fungal isolates out of 57 fungal isolates 51 isolate had MIC≤2µg/ml.

TABLE 18: MINIMUM INHIBITORY CONCENTRATION FOR VORICONAZOLE - BROTH MICRODILUTION METHOD

Organism	0.25 µg	0.5 µg	1 µg	2 µg	4 µg	8 µg	16 µg	32 µg	64 µg
Aspergillus Flavus	2	3	4	5	-	-	-	-	-
Aspergillus niger	2	3	4	3	-	-	-	-	-
Aspergillus Fumigatus	2	3	3	1	1	-	-	-	-
Fusarium spp	3	2	3	4	-	-	-	-	-
Curvularia spp	-	-	1	1	-	-	-	-	-
Penicillium spp	-	1	1	1	-	-	-	-	-
Graphium spp	-	-	1	1	-	-	-	-	-
Scopulariopsis spp	-	1	1	-	-	-	-	-	-

One isolate of *Aspergillus Fumigatus* which had MIC of 4µg/ml. All the other fungal isolates had of MIC≤2µg/ml by Broth Microdilution method for Voriconazole.

FIGURE - 12

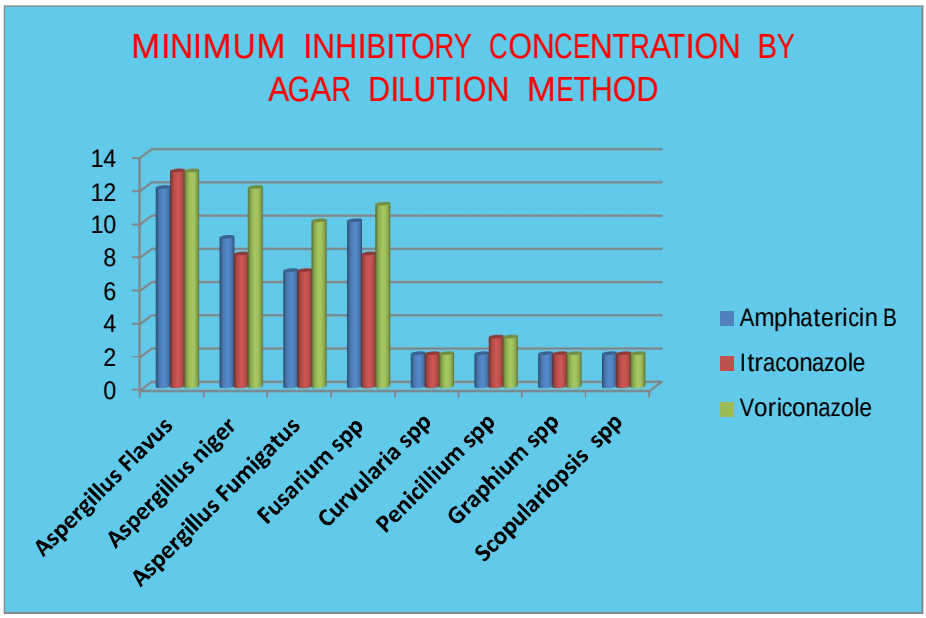


FIGURE - 13

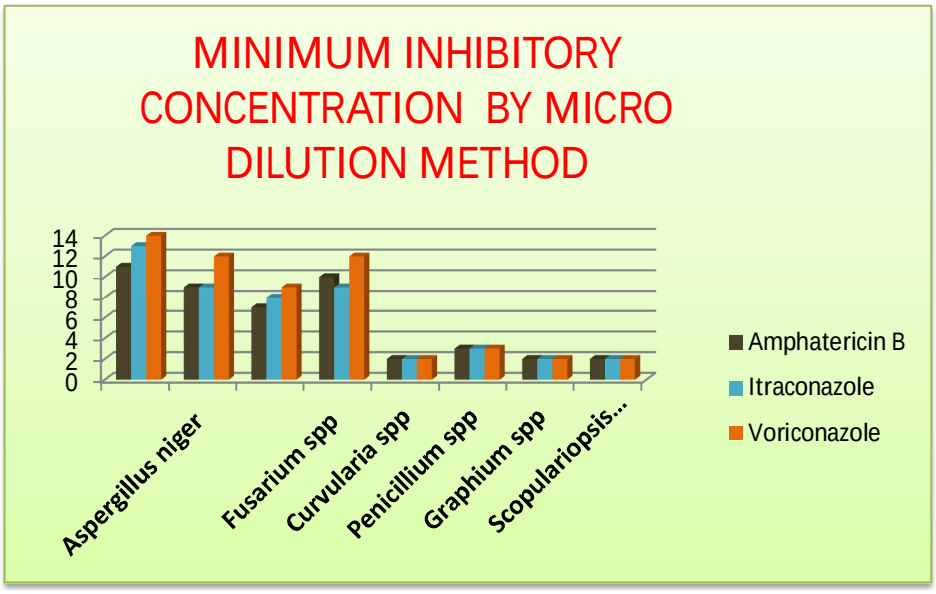


TABLE 19: COMPARISION OF MIC IN AGAR DILUTION AND BROTH MICRODILUTION

Drug Concentration	Amphatericin B MIC < 2 µg		Itraconazole MIC < 2 µg		Voriconazole MIC<2µg	
Organism	Agar Dilution Method	Broth Microdilution Method	Agar Dilution Method	Broth Microdilution Method	Agar Dilution Method	Broth Microdilution Method
Aspergillus Flavus	12	11	13	13	13	14
Aspergillus niger	9	9	10	10	12	12
Aspergillus Fumigatus	7	7	7	8	10	9
Fusarium spp	10	11	10	11	11	12
Curvularia spp	2	2	2	2	2	2
Penicillium spp	2	3	3	3	3	3
Graphium spp	2	2	2	2	2	2
Scopulariopsis spp	2	2	2	2	2	2
Total	46	47	49	51	55	56
Percentage	80.70%	82.45%	85.96%	89.47%	96.49%	98.24%

A good correlation were observed between agar dilution method and broth micro dilution method in the sensitivity pattern of fungal isolates with antifungal drugs. On comparison of both the methods Broth micro dilution method was found to be sensitive than the Agar dilution method.

TABLE 20: EVALUATION OF 10% POTASSIUM HYDROXIDE MOUNT SCREENING TEST

10% KOH Mount	Culture		Total
	Positive	Negative	
Positive	50	2	52
Negative	4	94	99
Total	**54**	**96**	**150**

Sensitivity = TP/ (TP+FN) = 92.59%

Specificity = TN/(TN+FP) = 97.91%

10% KOH mount was found to be sensitive and specific for preliminary diagnosis of fungal keratitis.

RESULTS

In this study, 150 cases of traumatic infectious keratitis patients, 94 cases were culture positive (Table 1).

The cases were analyzed under the following parameters.

The age and sex distribution of infectious keratitis was analysed 28.72% of infectious keratitis case were in 51-60 years of age group. Extremes of Age group showed low prevalence (<10 and >70%) (Table 3).

In this study male predominated in all forms of keratitis 71.11% (90/150) were males, and 50% (30/150) were females. (Table 2)

The urban and rural distribution of cases showed higher prevalence of infectious keratitis in rural population accounting for 64.66% (Table 4)

In analyzing the contribution of different trauma factors in infectious keratitis, trauma with vegetable matter like paddy, leaf, wood were implicated in 47% cases (Table 6).

Majority of the aetiological agents were fungi (57.44%) followed by bacteria (32.97%), mixed bacteria and fungal growth was observed in (3.19%) of the cases. The parasitic growth was observed in 6.38% of the cases (Table 5).

Among the fungal isolates, 33 out of 54 (63.15%) cases were due to *Aspergillus spp*. The next common agent was *Fusarium spp* 12 out of 54 (21.05%), followed by *Pencillium spp* 3 out of 54 (5.26%) *Graphium spp* 2 out of 54 (3.50%), and *Scopulariopsis spp* 2 out of 54 (3.50%).(Table 8)

Staphylococcus aureus was the most common bacterial isolate, accounting for 26.47%, followed by *Pseudomonas aeruginosa* (17.64%) *Acinetobacter boumannii* was isolated in 4 cases (11.76%), *Staphylococcus epidermidis* (17.64%), *Klebsiella pneumoniae* (16.70%), *Escherichia coli* (11.76%) (Table 7).

The sensitivity pattern of bacterial isolates revaled sensitivity to amikacin 96.11%, 44.11 to gentamicin, 82.33 to ciprofloxacin, 61.73% to ofloxacin, 79.41% to cefataxime, 26.47% to cotrimoxazole, 76.47% to cephalexin, 100% to vancomycin, and 32.35% to oxacillin (Table 10).

Two *Staphylococus aureus* isolates exhibited to oxacillin resistant and both isolates were sensitive to vancomycin (Table 11)

Antifungal sensitivity pattern of fungal isolates by disc diffusion test showed that 64% (9) *Aspergillus flavus,* 58.3% (7) *Aspergillus niger* isolates, 70% of (7) *Aspergillus fumigatus* isolates and 66% (8) of *Fusarium spp*, 50% (1) of *Curvularia spp,* 33% (1) of *pencillium spp,* 50% (1) of *Graphium spp,* and 50% (1) *Scopulariopsis spp* were sensitive to Amphotericin B (Table 12)

Itraconazole was sensitive in 78% (9) of *Aspergillus flavus*, 100% (12) of *Aspergillus niger* isolates, 100% of (10) *Aspergillus fumigatus* isolates and 83% (10) of *Fusarium spp,* 50% (1) of *Curvularia spp*, 66% (2) of *pencillum spp*, 100% (2) of of *graphium spp* and 100% (2) of *Scopulariopsis spp* were sensitive to Amphotercin B (Table 12)

10% Potassium hydroxide mount preparation used as screening test for rapid diagnosis of infectious keratitis showed sensitivity 92.59% and 97.91% specificity (Table 20)

MIC determination by broth microdilution method also showed that MIC range was comparable with Agar dilution method.

84.48% of *Aspergillus spp* and 81% of *Fusarium spp*, 100% of *Curvularia, Graphium spp, Scopulariposis spp* and 60.66% of *Pencillium spp* showed sensitive range of Amphotercin B by Agar dilution method(Table 12).

Itraconazole (99.9%) of *Aspergillus spp* and 83.3% of Fusarium isolates, 100% of *Curvularia spp, Graphium spp, Scopulariopsis spp*, *Pencillium spp* showed MIC<2 μg/ml. (Table 14)

Voriconazole was 96.41% sensitive in Agar dilution methods, and 98.2% in Broth Microdilution Method. (Table 19)

COLOUR PLATES

PLATE NO.1 A CASE OF FUNGAL KERATITIS

PLATE NO.2 COLLECTION OF CORNEAL SCARPINGS

PLATE NO.3 10% KOH MOUNT – HYPHAL ELEMENTS

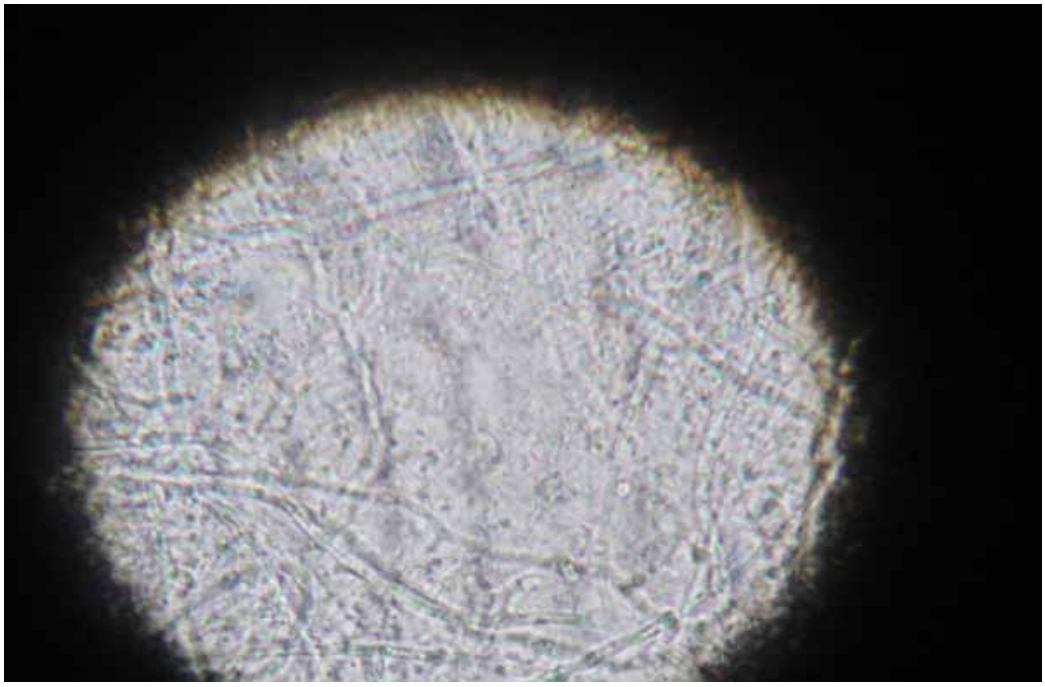


PLATE NO.4 CORNEAL SCRAPING – LPCB MOUNT

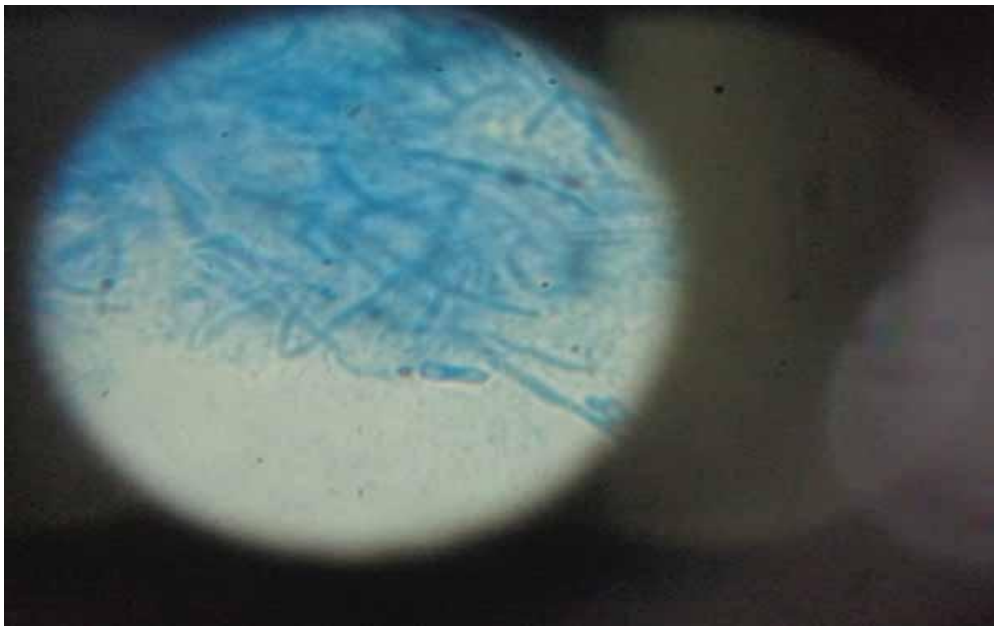


PLATE NO.5 CORNEAL SCRAPING – GIEMSA STAIN

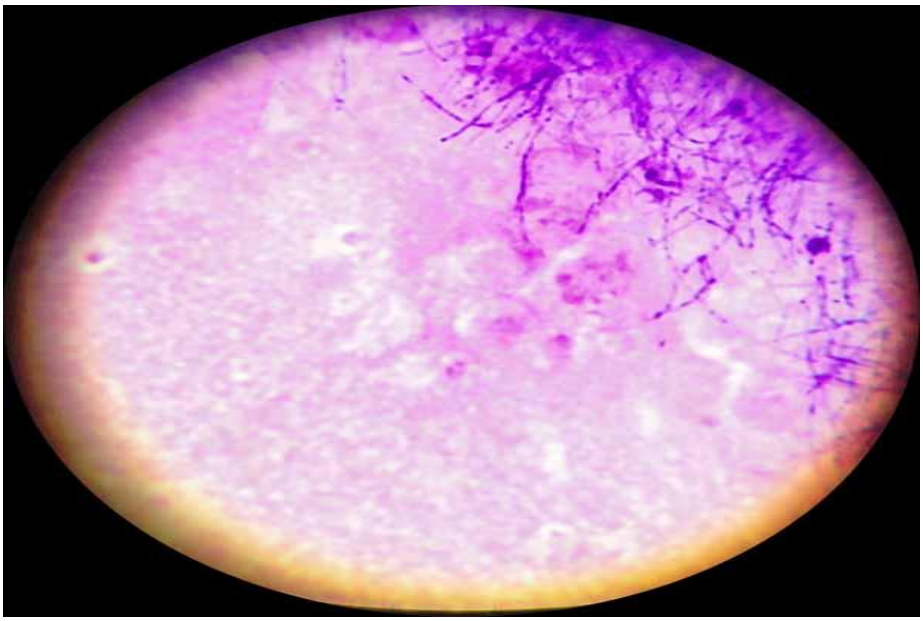


PLATE NO.6 GIEMSA STAIN – HYPHAL ELEMENTS

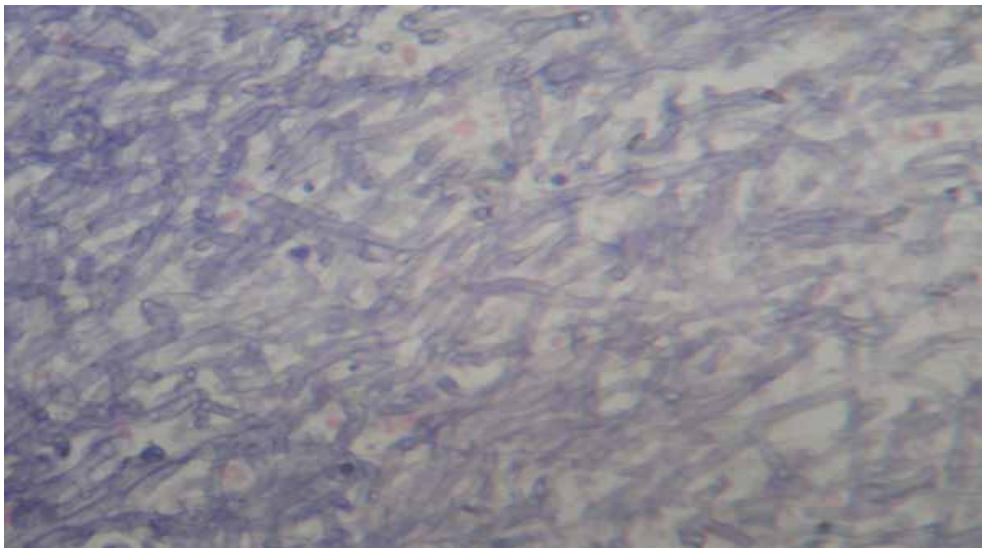


PLATE NO. 7 ASPERGILLUS FUMIGATUS IN – SDA PLATE

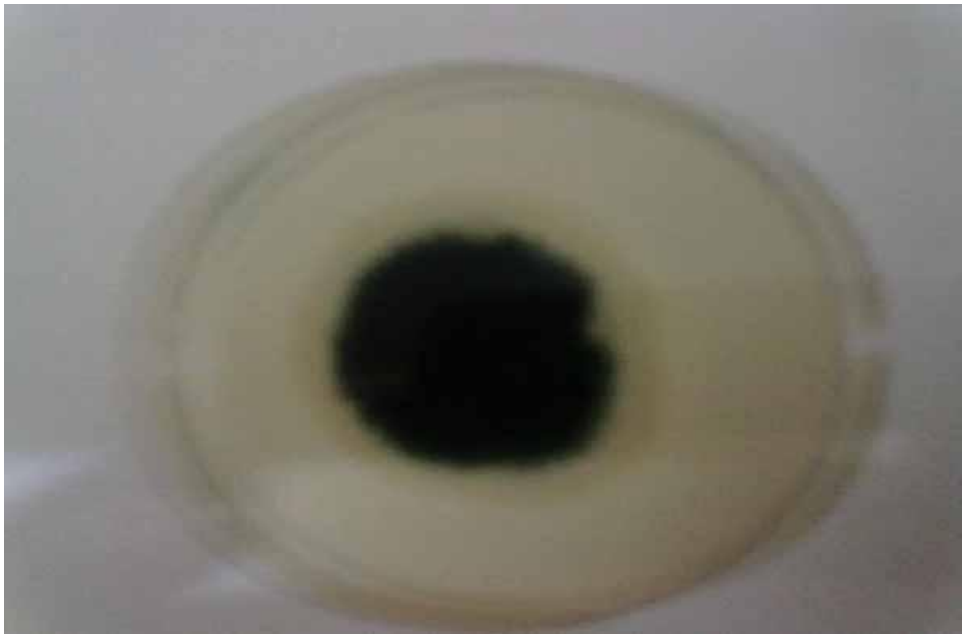


PLATE NO.8 ASPERGILLUS NIGER - LPCB MOUNT

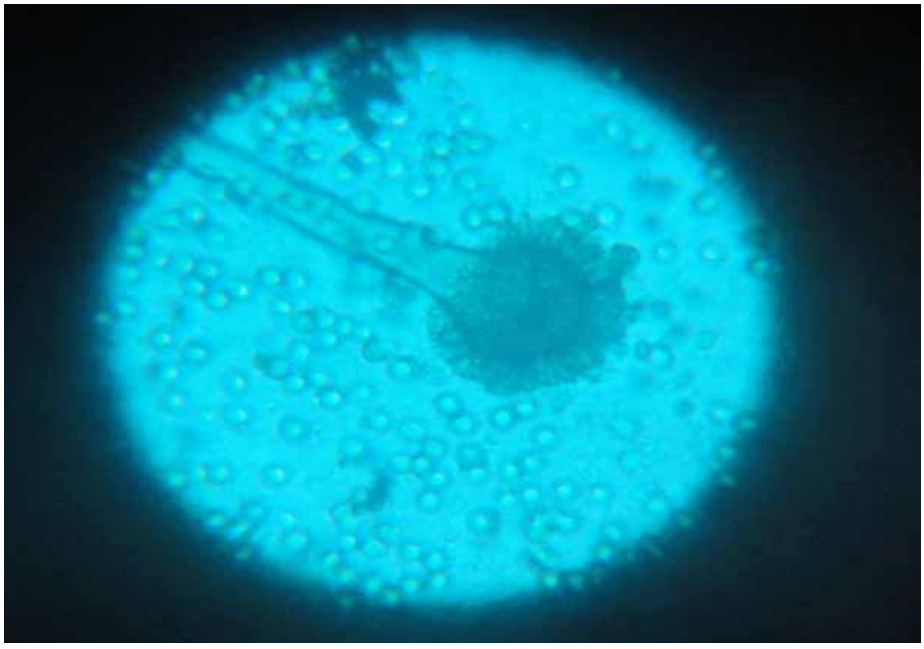


PLATE NO.9 ASPERGILLUS FUMIGATUS - LPCB MOUNT

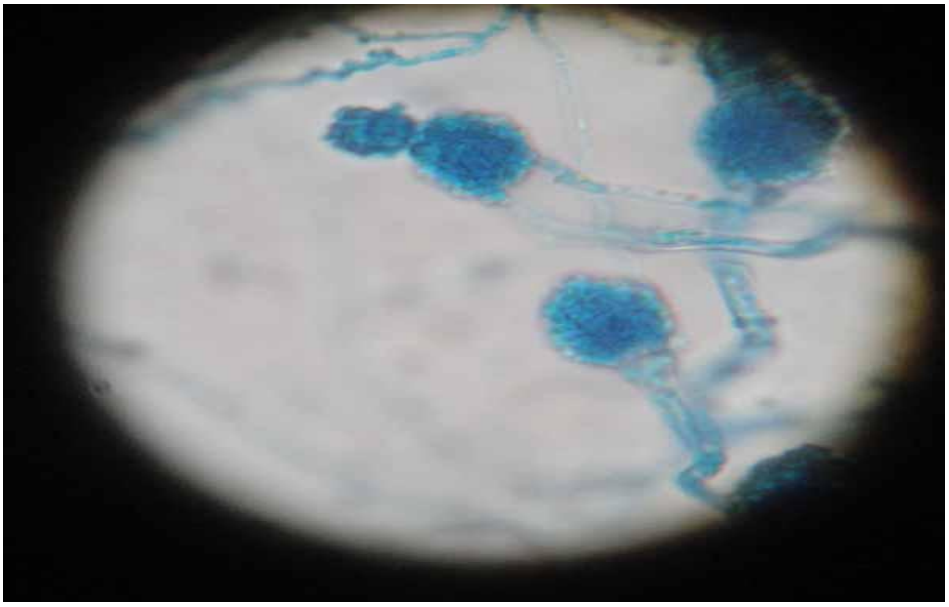


PLATE NO.10 ASPERGILLUS FLAVUS- SDA PLATE

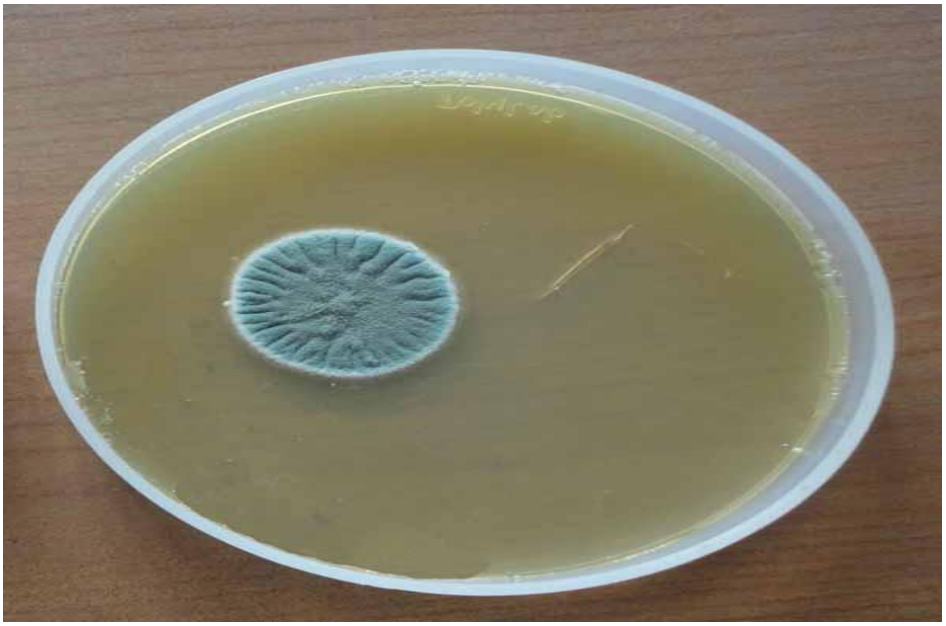


PLATE NO.11 CALCOFLOUR STAIN SEPTATE HYPHAL ELEMENTS

PLATE NO.12 ASPERGILLUS FLAVUS- LPCB MOUNT

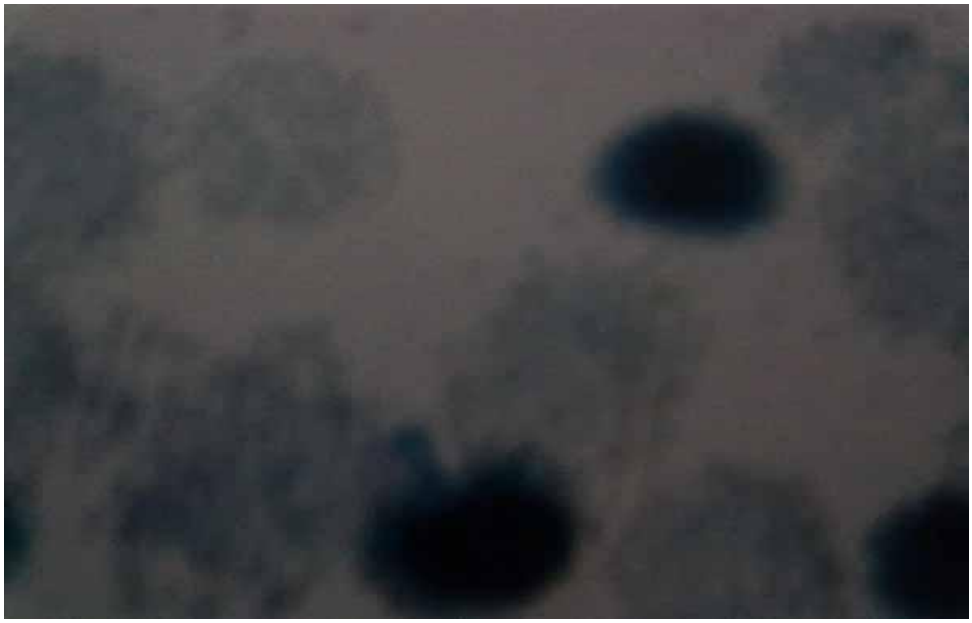


PLATE NO.13 FUSARIUM SPP - LPCB MOUNT

PLATE NO.14 PENCILLIUM SPP - LPCB MOUNT

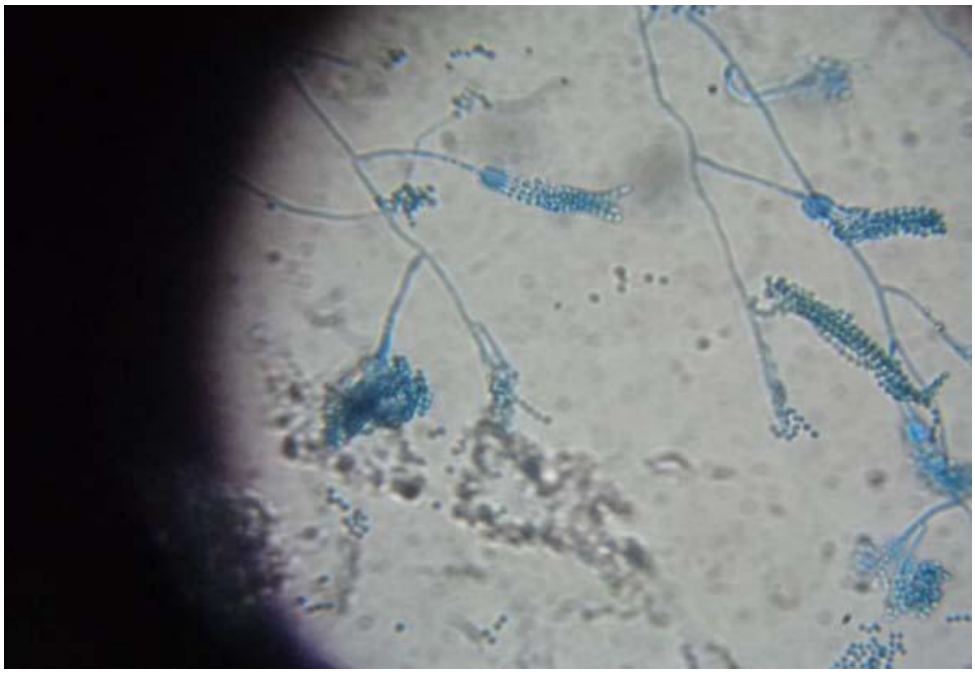


PLATE NO.15 CURVULARIA SPP - LPCB MOUNT

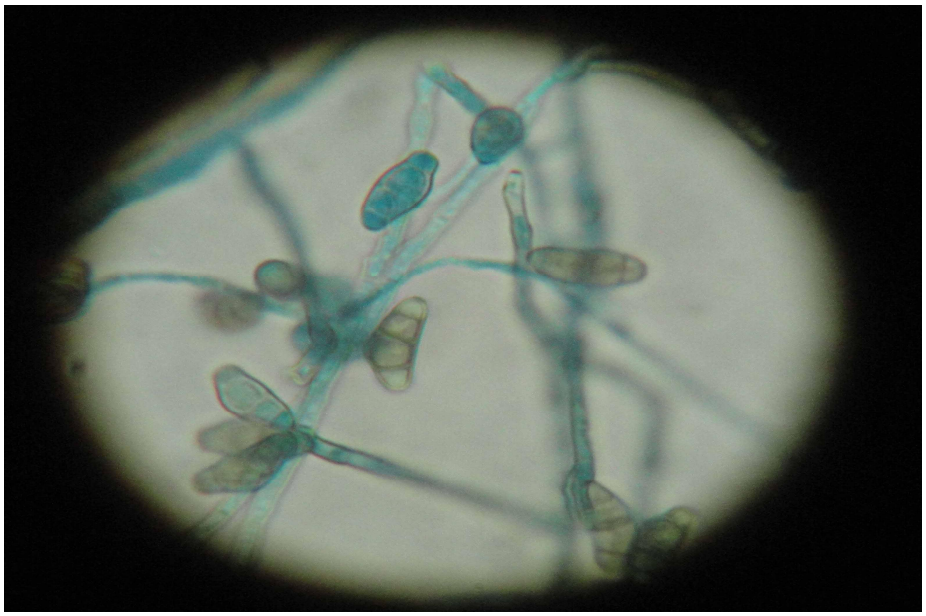


PLATE NO.16 SCOPULARIOPSIS SPP- LPCB MOUNT

PLATE NO.17 GRAPHIUM SPP - LPCB MOUNT

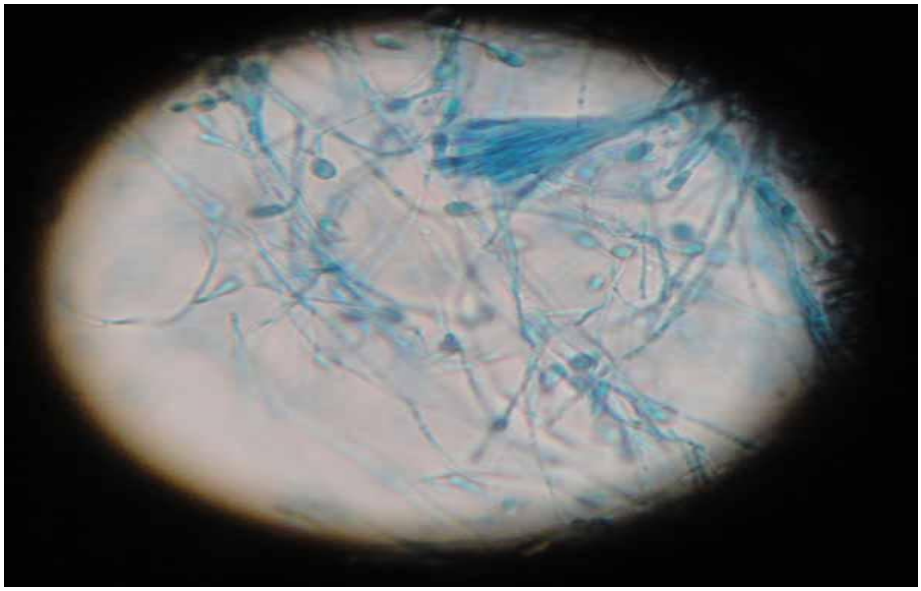


PLATE NO.18 GRAPHIUM SPP CULTURE TUBES

PLATE NO.19 CLEISTOTHECIUM - PSEUDALLESCHERIA BOYDI - LPCB MOUNT

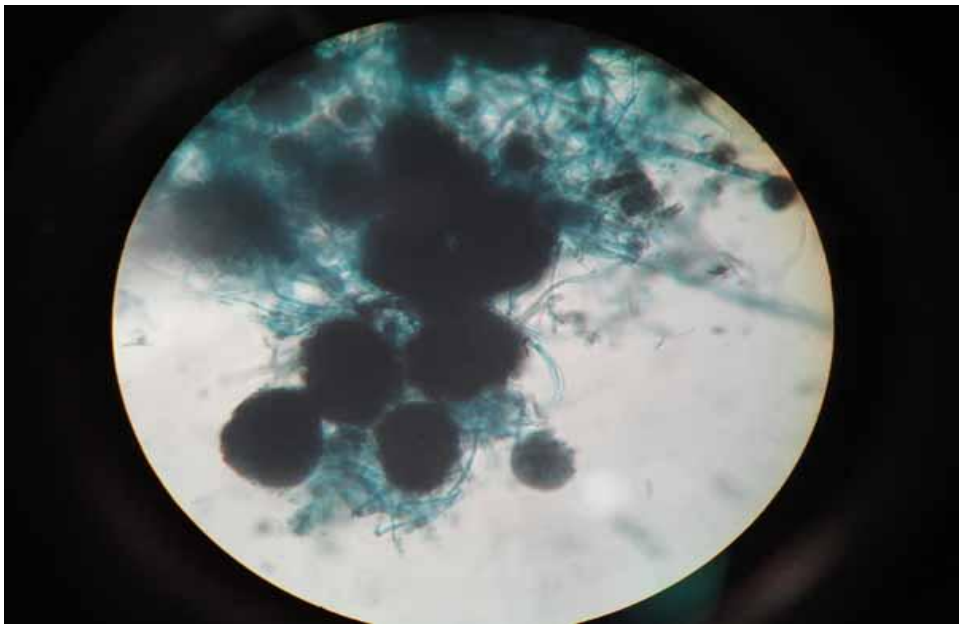


PLATE NO.20 CLEISTOTHECIUM - PSEUDALLESCHERIA BOYDI- WET MOUNT

PLATE NO.21 ANTIFUNGAL SUSCEPTIBILITY – DISC DIFFUSION METHOD(ASPERGILLUS FLAVUS)

PLATE NO.22 ANTIFUNGAL SUSCEPTIBILITY – DISC DIFFUSION METHOD (ASPERGILLUS NIGER)

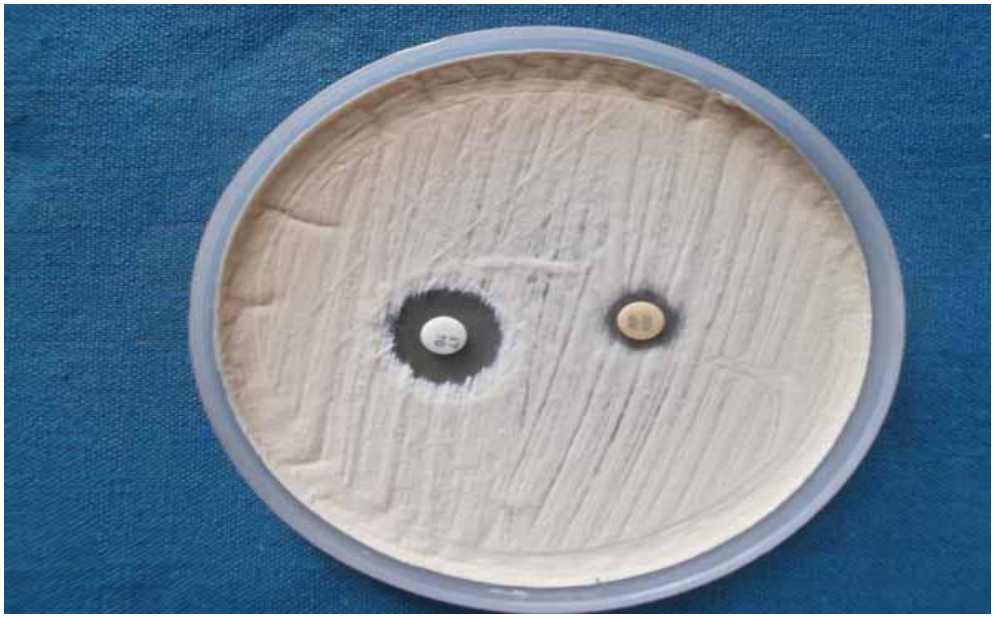


PLATE NO.23 ANTIFUNGAL SUSCEPTIBLITY TESTING - MIC BY E - STRIP METHOD

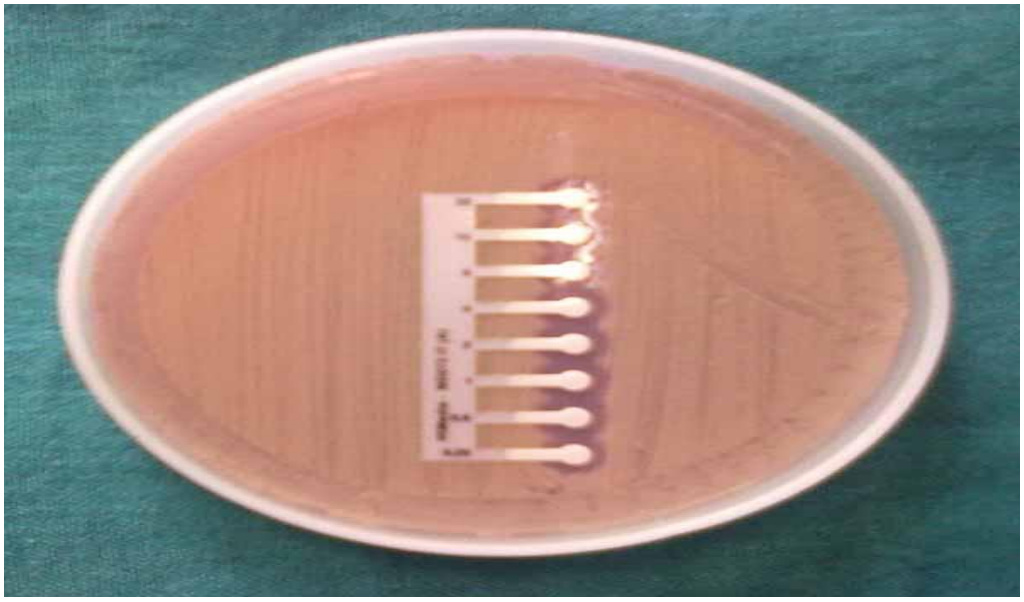


PLATE NO.24 ANTIFUNGAL SUSCEPTIBILITY – BROTH MICRO DILUTION METHOD AMPHOTERICIN B

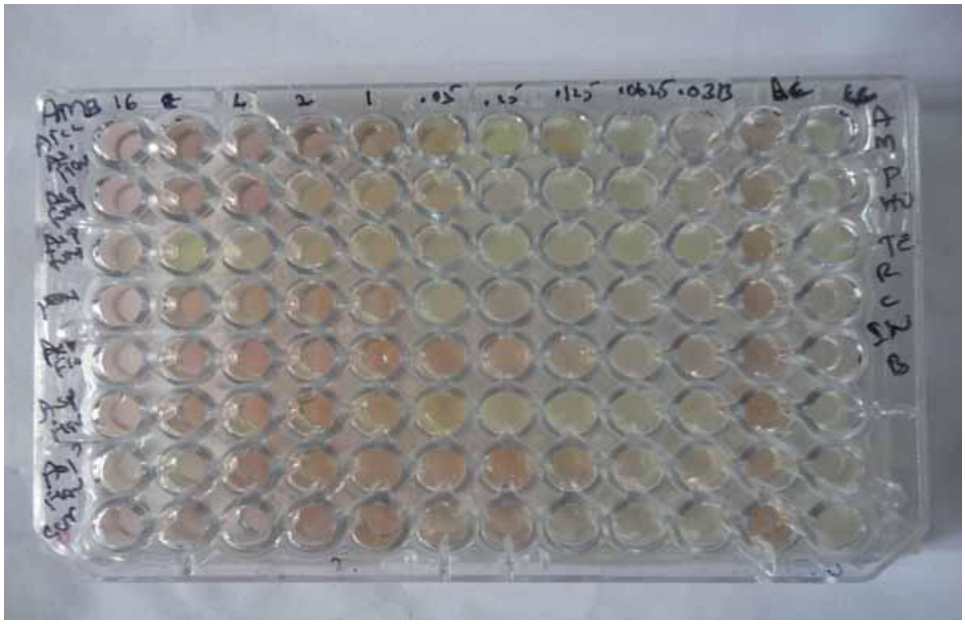


PLATE NO.25 ANTIFUNGAL SUSCEPTIBILITY – BROTH MICRO DILUTION METHOD VORICONAZOLE

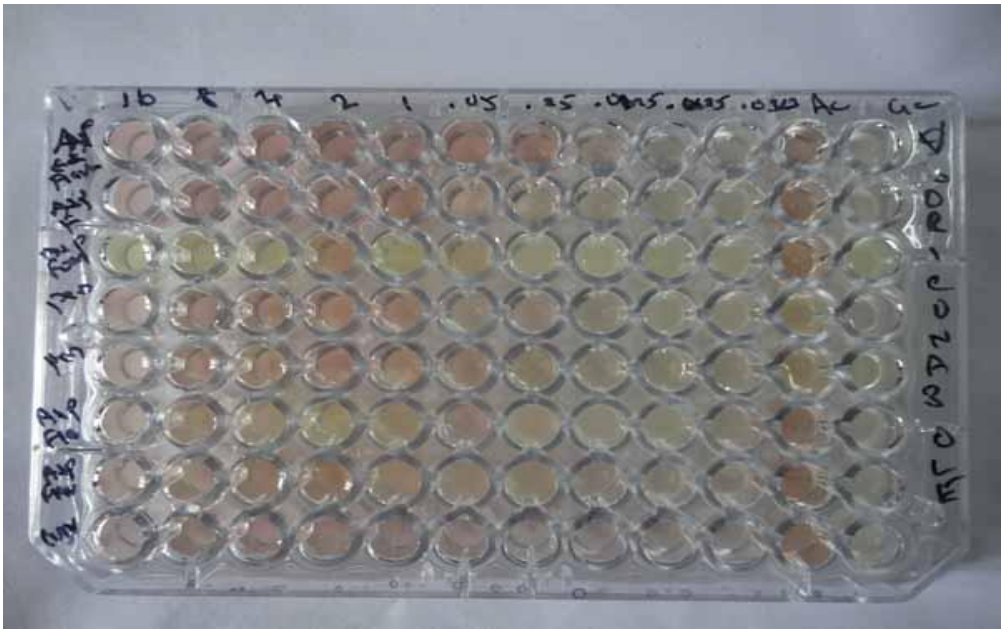
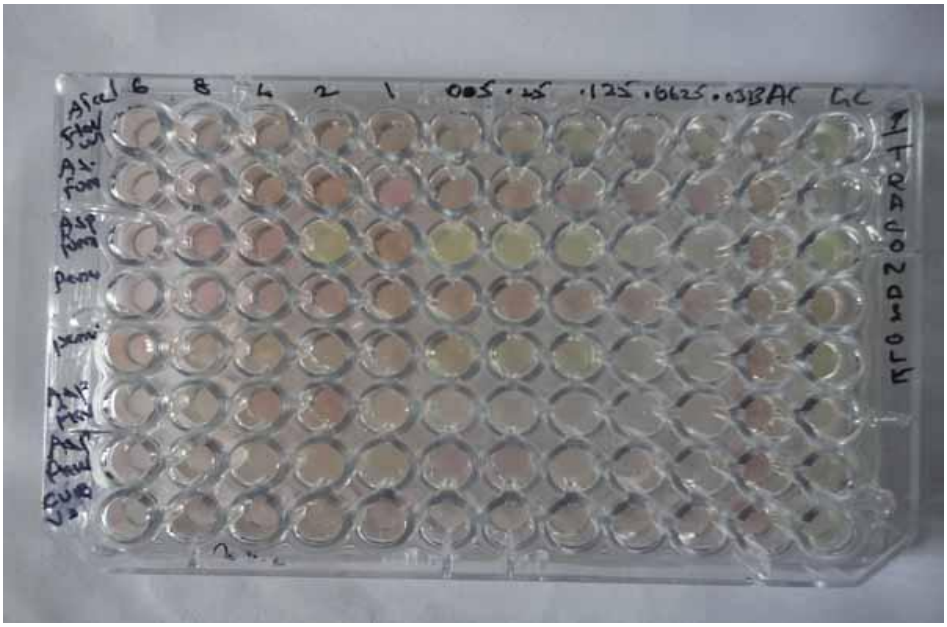


PLATE NO.26 ANTIFUNGAL SUSCEPTIBILITY – BROTH MICRO DILUTION METHOD ITRACONAZOLE



BACTERIAL ISOLATES

PLATE NO.1 A CASE OF BACTERIAL KERATITIS

PLATE NO.2 C STREAK METHOD – BLOOD AGAR PLATE

PLATE NO.3 GRAM STAIN – STAPHYLOCOCCUS AUREUS

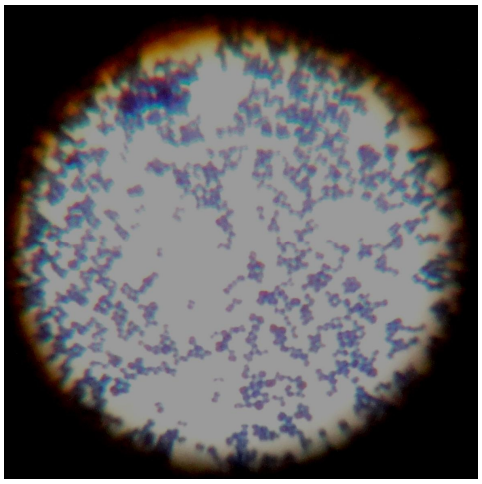


PLATE NO.4 TUBE COAGULASE TEST

PLATE NO.5 DNASE TEST –STAPHYLOCOCCUS AUREUS

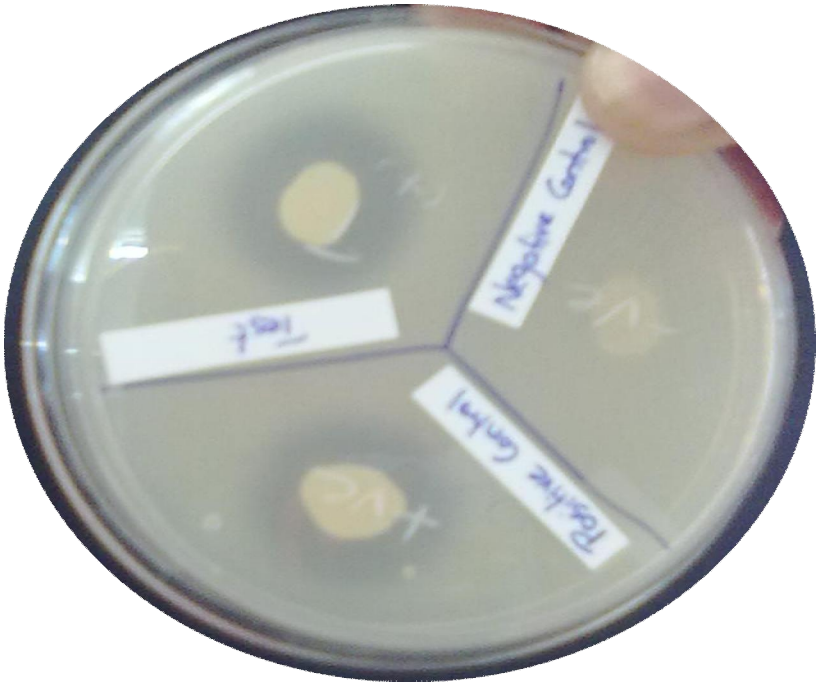


PLATE NO.6 BIO CHEMICAL REACTION - STAPHYLOCOCCUS EPIDERMIDIS

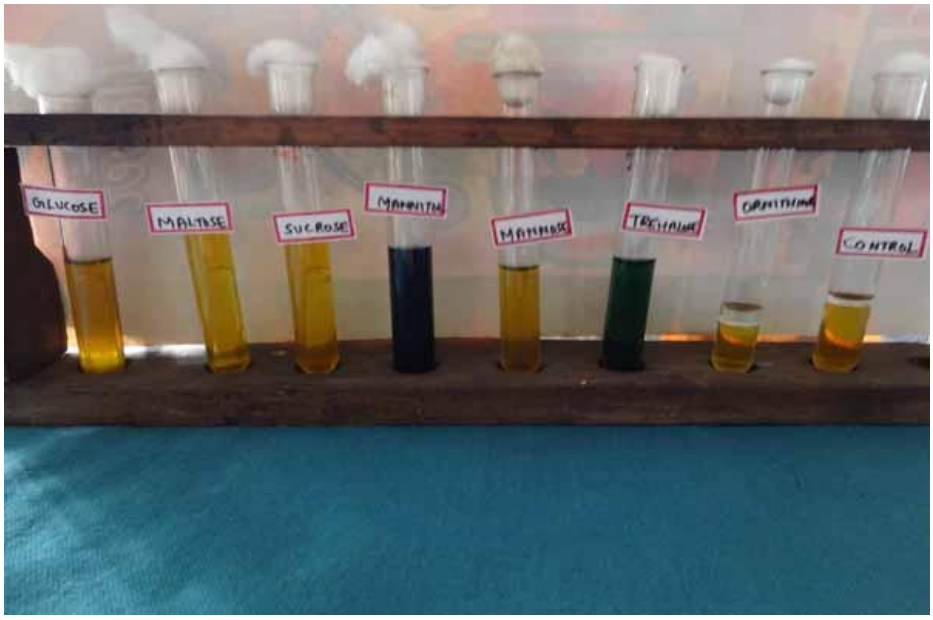


PLATE NO.7 BIOCHEMICAL REACTIONS - E COLI

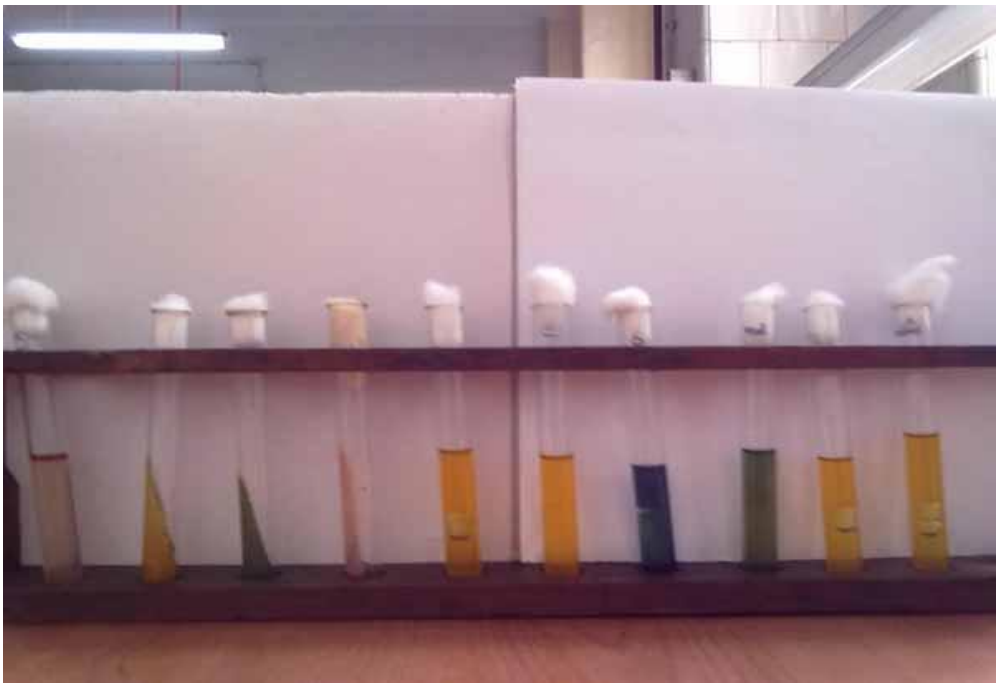


PLATE NO.8 ANTIMICROBIAL SUSCEPTIBILITY OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS

PLATE NO.9 ANTIBIOGRAM –PSEUDOMONAS AERUGINOSA

PLATE NO.10 ESBL - PHENO TYPING CONFIRMATION METHOD

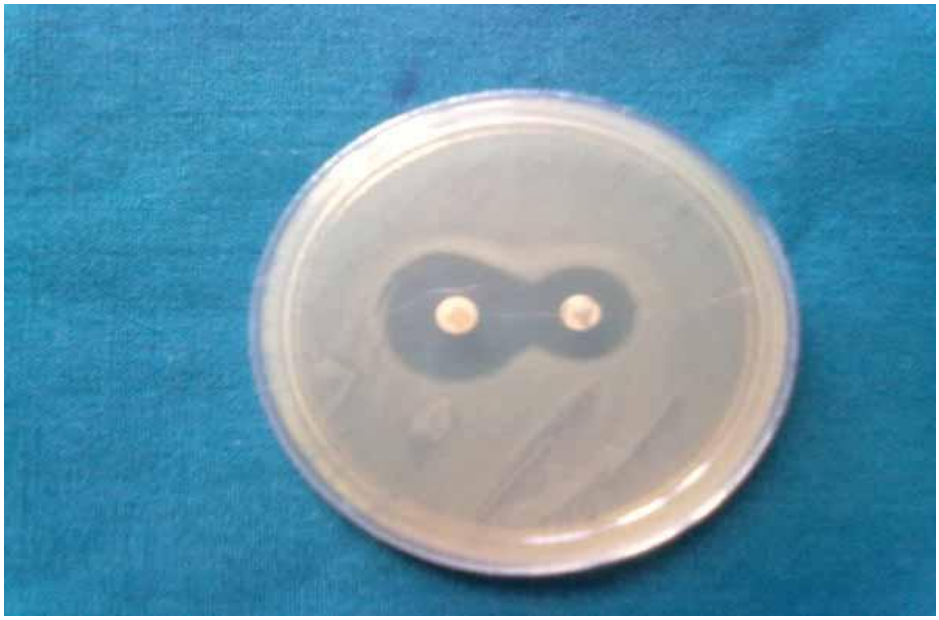


PLATE NO.11 ESBL DETECTION - DOUBLE DISC METHOD

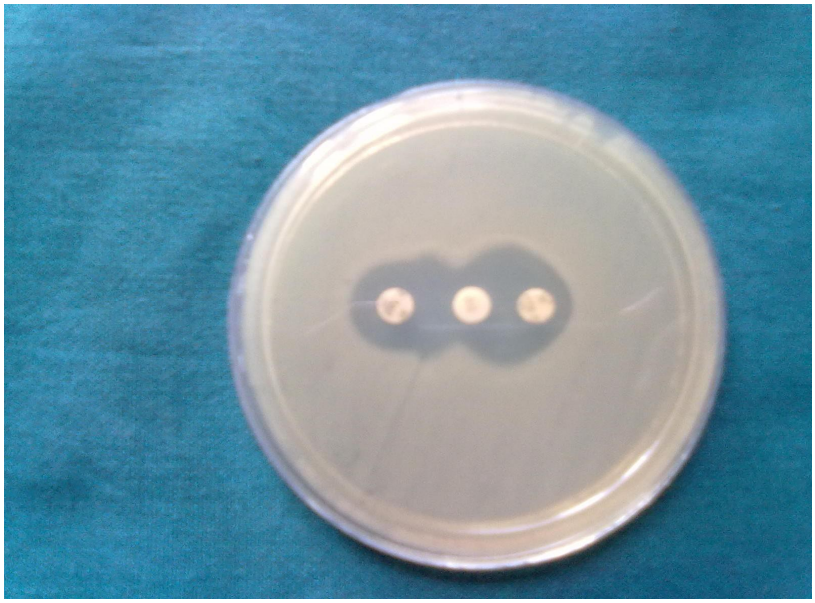


PLATE NO.12 ESBL DETECTION BY E STREIP METHOD

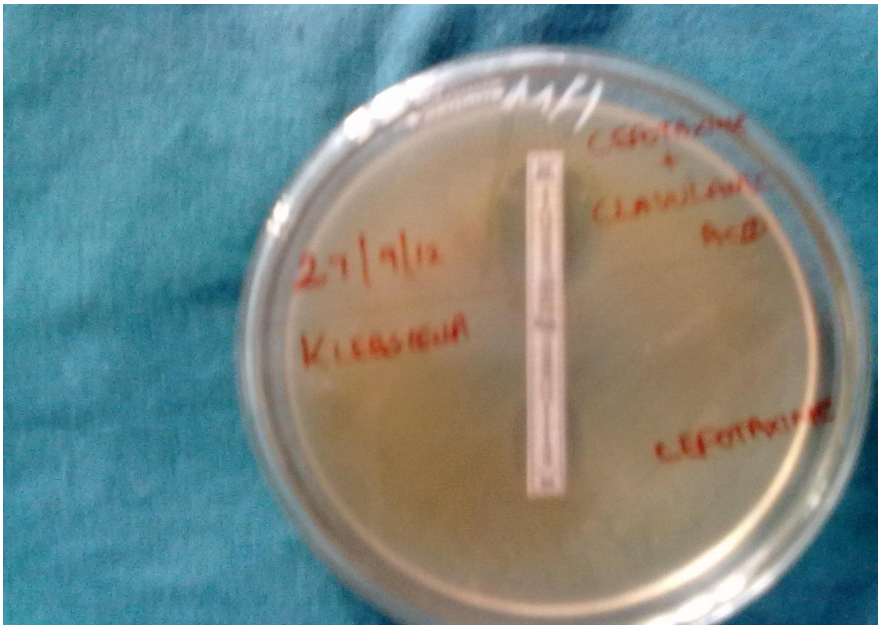
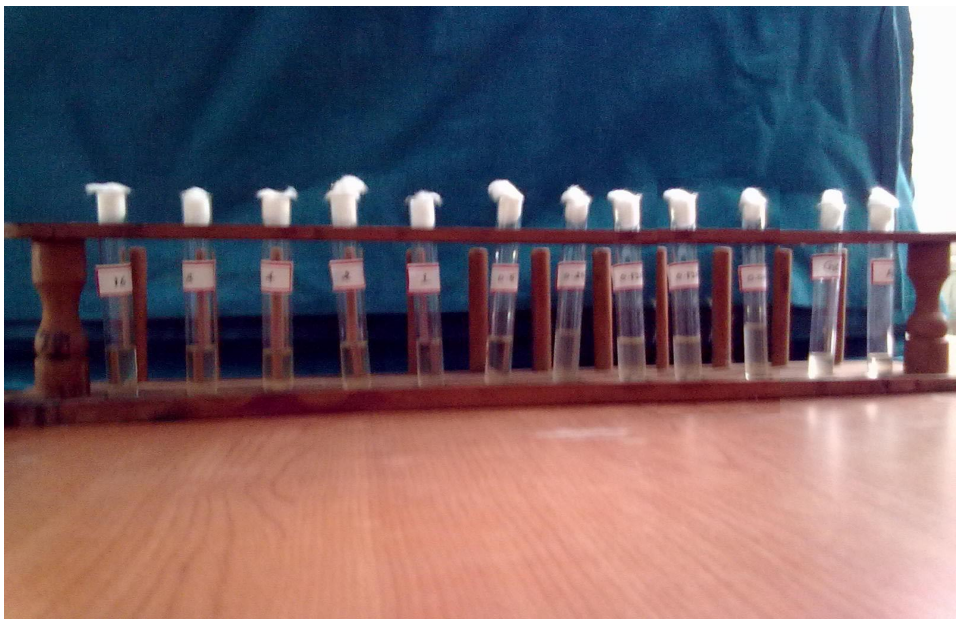


PLATE NO.13 MIC OF VANCOMYCIN FOR STAPHYLOCOCCUS AUREUS



PARASITIC ISOLATES

PLATE NO.1 A CASE OF ACANTHAMOEBA KERATITIS

PLATE NO.2 10% KOH MOUNT - ACANTHAMOEBA CYST

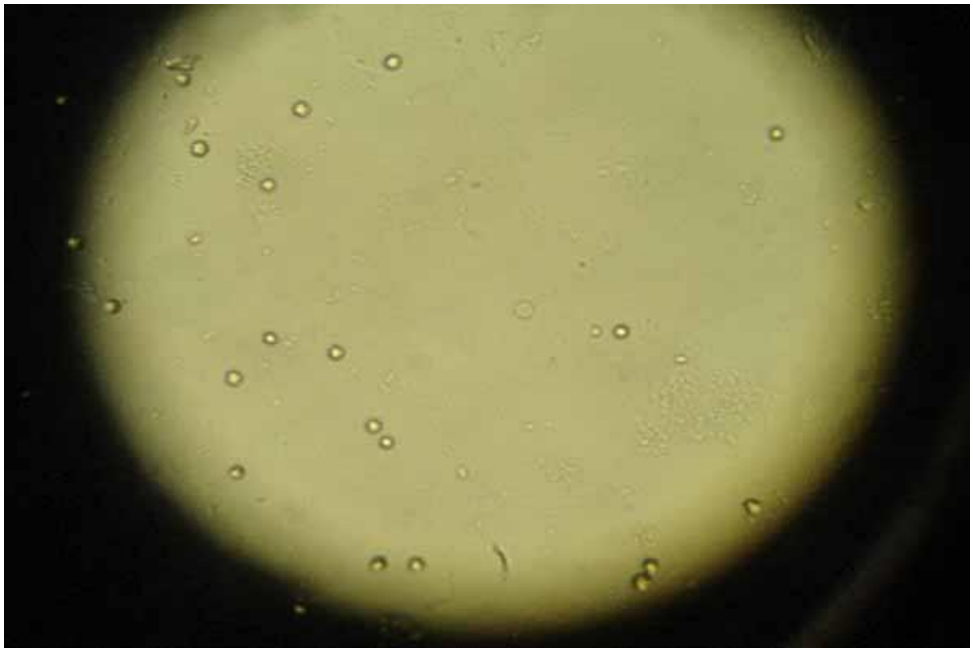


PLATE NO.3 GRAMS STAIN ACANTHAMOEBA CYST

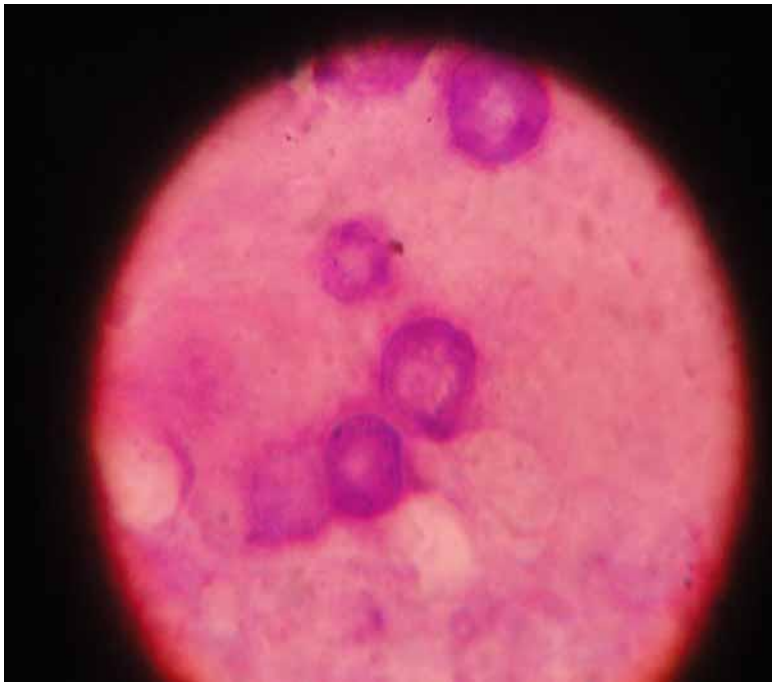


PLATE NO.4 CULTURE PLATE ACANTHAMOEBA CYST

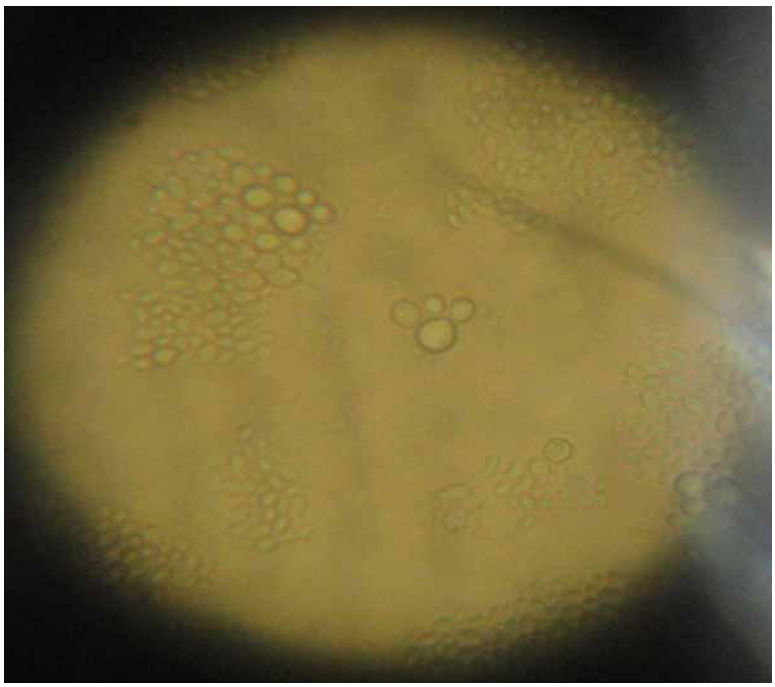


PLATE NO.5 CULTURE PLATE ACANTHAMOEBA CYST

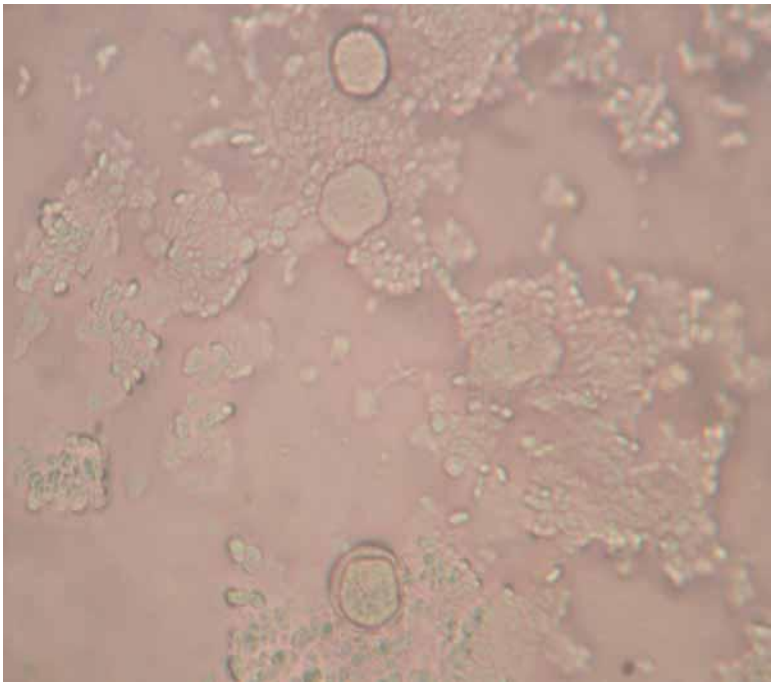


PLATE NO.6 LPCB MOUNT ACANTHAMOEBA CYST

PLATE NO.7 WET MOUNT ACANTHAMOEBA CYST

PLATE NO.8 CALCO FLOUR STAIN
ACANTHAMOEBA CYST(10 X)

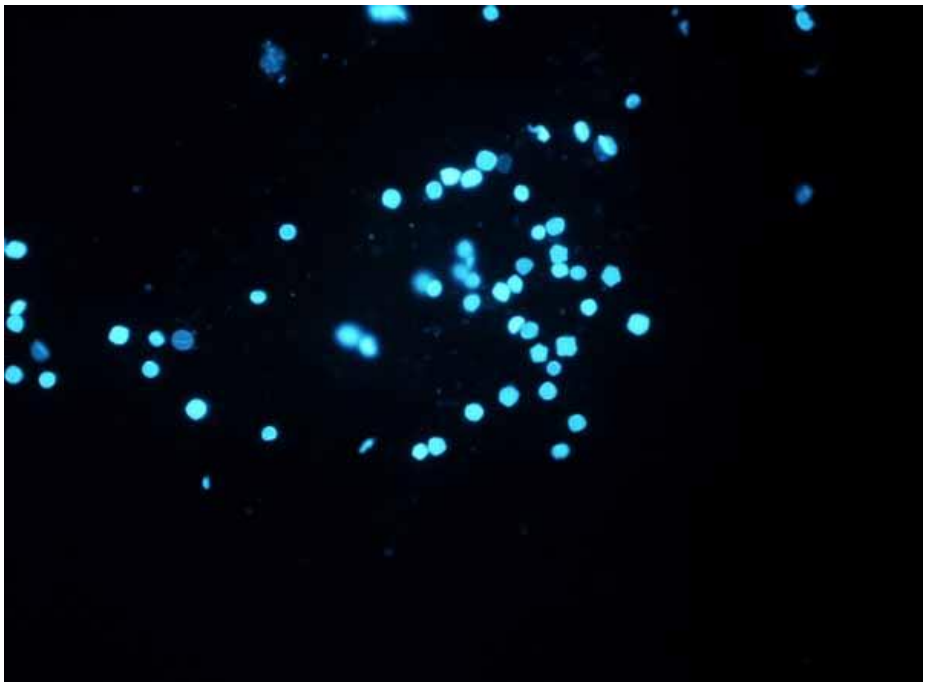
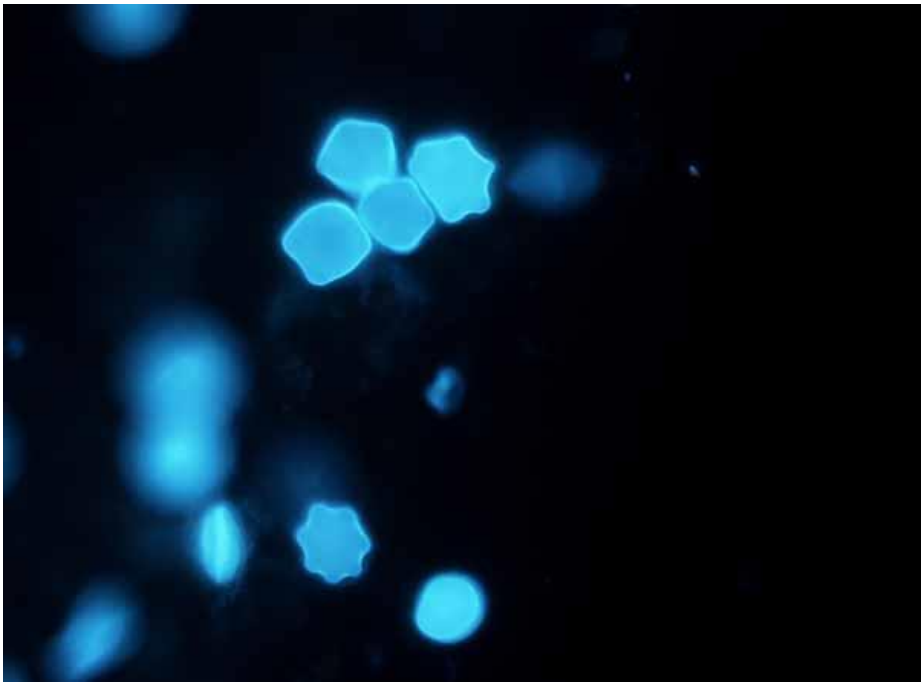


PLATE NO.9 CALCO FLOUR STAIN
ACANTHAMOEBA CYST(40 X)

PLATE NO.10 CALCO FLOUR STAIN
ACANTHAMOEBA CYST



DISCUSSION

DISCUSSION

Corneal disease, especially infectious keratitis, is a major cause of vision loss and blindness second only to cataract.[105] A study from South India reported that the incidence of corneal infections in India is almost 10 times higher than that reported in the United States.[104]

Infectious keratitis may be caused by bacteria, fungi, protozoa and virus and the spectrum of microbial pathogens causing keratitis varies according to the geographical locations and climatical conditions. [8,36,105]

The present study showed the following results. Out of 150 cases of traumatic infectious keratitis studied, 94 cases showed culture positivity which accounts for 62.66%.(Table 1) The culture positivity was similar to the Basak *et al,* 1991 study which showed 65.4% of corneal injury cases having microbial keratitis. [4]

Upadhyay MP *et al,* study also showed 55% in North India, and 52.8% in Nepal as corneal trauma cases having microbial keratitis. [101]

M.Jayanth Bharathi *et al,* 2009 study showed that corneal injury was responsible for 71.5% cases of corneal ulcer.[7] This study

was also similar to the study of M.Srinivasan *et al*,1993 from Madurai which showed (68.4%) positivity[94] and Geetha K.V. *et al* in 2002 which showed 78% culture positivity.[29]

Culture negativity was reported in 44% cases in the present study. This coincides with Bharathi *et al*[9] 29.41% and Mohapatra *et al*[69] 32.3%, Gita *et al*[30] reported a higher incidence of culture negativity 62%, 23 out of 44 culture negative cases had a history of prior antibiotic and anti fungal drug usage before attending to the hospital. Culture negativity could be due to failure to obtain material from the proper site or failure to reach deep enough to obtain scrapings containing the pathogens and may be due to viral aetiology.

COMPARISON OF CULTURE POSITIVITY WITH OTHER STUDIES

Author	Bacterial %	Fungal %	Acanthamoeba %	No Growth %
Zhang *et al* (2002) China	18	34.8	2.4	44.80
Bharathi *et al* (2003) S.India	32.77	34.4	1.04	29.41
Mohapatra *et al* (2003) Calcutta	49.05	18.73	-	32.2

Author	Bacterial %	Fungal %	Acanthamoeba %	No Growth %
Gita *et al* (2004) San Francisco	27	8	3	62
Jayhar M, Bharath *et al* (2009)	33	32	2.5	29

Present study showed that males were more commonly affected than females (71.11%). Male preponderance may be due to occupation, eg. Agricultural workers are more prone to corneal injury and were washing eyes with probably stagnant water.[28,108] This study was similar to Bharathi *et al,*2003 from South India who reported 56.76% of corneal ulcer in males[9]. Gopinath *et al,*2002 from Hyderabad study also showed that 71.1% of corneal ulcer in males,[9] and Jothi Padmaja *et al,*1990 from Visakhapatnam showed 68.58% in males with corneal injury due to trauma.(Table 2) [49]

This study is also similar to Chowdhary *et al,*2005, which showed higher incidence (68%) among males.[19] Reema nath *et al,* in 2011 from upper Assam study also showed higher incidence of corneal ulcer in male patients 67.6%.[81]

People of certain age group are affected more in infectious keratitis.[57] In this study, most common age group affected was between 51-60 years (28.72 % - Table 3). This study was similar with the study of Bharathi MJ, *et al* 2003.[11] The study of Chander J *et al* 1994, also showed more number of patients with infectious keratitis in the 51-60 years age group.[15] Manikandan P *et al*,2004 study also showed that patients above 50 years of age were more vulnerable to microbial keratitis.[66]

In this study, rural population were more vulnerable to infectious keratitis due to trauma compared to the urban population (64.66% - Table 4). This present study was similar to Basak samara *et al***,** in 2005[85] from West Bengal showed that 78.5% of the patients were from rural areas.[4]

Bharathi MJ *et al*,2003[8] and Chandar J *et al*, [16] 1994 also showed higher incidence of infectious keratitis from rural areas.

The present study showed that different agents such as paddy dust, wood, Insect fall, stick, Cow's tail, thorn prick, stone, iron particles were the etiological agent casing corneal injury leading to infectious keratitis. (Table 6)

In this study corneal injury with vegetable matter was the

most common factor casing infectious keratitis (47.87% - Table 6) This study was similar to Basak Samar *et al,* in 2005 West Bengal who reported (59.6%) traumatic cases due to vegetable matter.[4] Norina TJ, *et al*, 2008 and Laspinal F,*et al*, 2004,[59] also showed history of ocular trauma in 62% and 50% of their patients respectively.

The above observation clearly show that in developing countries like India where, agricultural worker were more common, vegetative matter induced ocular trauma was the major cause of infectious keratitis in rural population than urban population. [22,31,80]

In this present study 5 (3.3%)patient were found to have Diabetes mellitus. This study was similar to Basak samara K, *et al,*2005, study where 7.6% of patients had Diabetes mellitus.[4] Gopinath *et al*, 2002 from Hyderabad showed Diabetes mellitus as the predominant systemic disease associated with fungal and bacterial keratitis.[34]

In this study, aetioloigal agent was identified and isolated in 94 samples (62.66%). Of these 94 culture positive samples 54 (57%) had pure fungal growth, 31 (32.1%) had pure bacterial growth and 3 (3.19%) had mixed bacterial and fungal growth, 6

(6.38%) had parasitic growth. (Table 5)

In this study, fungal agents were the most common agent isolated in infectious keratitis patients followed by bacterial and parasitic agents.

This study correlates with the study of Basak Samar K *et al*, 2005,[4] which showed 62.7% of pure fungal growth, 22.7% of pure bacterial growth. This study was similar to Khanal B *et al*, in 2005, conducted in Nepal which showed 42.7% growth positive for fungi present.[52] This study also similar to Houang et al,2001.[35]

Aspergillus spp was the predominant isolate, in this present study (63.15%). This study was similar to Gopinath *et al*, 2002 from Hyderabad which showed that *Aspergillus spp* (30.7%) were the predominant isolate in their study.[34]

Basak Samar K *et al*, 2012 and Khanal B *et al* 2005 studies also showed that common fungal pathogen was *Aspergillus spp*, followed by *Fusarium spp*.[4,52,62]

The study done by Zimmerman E L *et al*, reported *Aspergillus spp* was the commonest fungal agent in corneal ulcer.[111]

In this present study *Fusarium spp* was found to be the next

common fungi isolated (21%). This study correlates with Gopinath *et al*,[32] 37.2% Mohapatra *et al*,[69] 2002 - 23% and Bharathi *et al*,[8] 2003 – 42.8% for *Fusarium spp.*

Reema Nath *et al.*, in 2011 study also showed that *Fusarium spp* was the most common species isolated from corneal ulcer.[81]

Lixen Xie, *et al*,[63] 2005 and Prashant Garg, *et al* 2000, studies also showed that *Fusarium spp* was found to be the most common fungi isolated. *Pencillium spp* was isolated in 5.26% of samples in the present study.

COMPARISON OF FUNGAL ISOLATES WITH INDIAN AUTHORS

Author	***Fusarium spp.%***	***Aspergillus spp%***	***Penicillium spp.%***	***Curvularia spp.%***
Jyothi Padmaja *et al* (1990) Visakhapatna	1.6	19.2	5.6	0.8
Panda *et al* (1997) Delhi	10.7	39.5	7	7.4
Verenkar *et al* (1998) Goa	12.5	61.2	12.5	6.3
Kumari *et al* (2002) Patna	7.89	52.26	7.89	2.8

Gopinathan *et al* (2002) Hyderabad	37.2	30.7	-	2.8
Mohapatra *et al* (2003) Delhi	23	38.4	-	-
Bharathi *et al* (2003) S.India	42.82	26	0.36	2.64

(- = Not reported)

In this present study 3.5% of *Graphium spp, 3.5% Scopulariopsis spp* were isoloated along with other fungal agent. 3.3% of *Curvularia spp* were isolated in this study, Which coincides with Bharathi *et al*,[8] 2003, 2.64% and Gopinath *et al*,[33] 2.8% 2002 Verenkar *et al*,[103] 1998, Panda *et al*,[77] 1997 studies also showed that higher incidence of 6.3% and 7.4% respectively. (Table 12)

Among the bacterial isolates *Staphylococcus aureus* 9 (27%) was the most common bacterial agent isolated. This study was similar to Basak samara k *et al*,[4] 2005, showed that *staphylococcus aureus* was the most common bacterial isolate.[7]

This study was also similar to Alexandar *et al*,[1] 19.4, Schaefer *et al*, - 22%, Zhang *et al*[89] – 21.7% and Gita *et al*,[30] 20% which showed that *Staphylococcus aureus* as the predominant bacterial isolate.

Pseudomonas aeruginosa was the next predominant isolate in the present study (17%). This study was similar to Basak Samar K *et al*[4] 2005 and Bharathi *et al*[6] 2003 which also showed 18.05 % for *Pseudomonas spp*. M.Srinivasan *et al*,[94] 1997 study also showed that *Pseudomonas spp*. 14.4% accounted for microbial causes for infectious keratitis. (Table 7) This study is also similar to Alexander *et al*, 2000 USA which showed 25.7% of *Pseudomonas spp* in their study.

Staphylococcus epidermidis were isolated in 18.18% cases in the present study. Alexandar *et al*,[1] 2000 and Gita *et al*,[30] also showed similar isolates.(Table 7)

Klebsiella spp 15.15%, *Escherichia coli* 12.12% cases were isolated in the present study,(Table 7) Which correlated with M.Jayahar Bharathi *et al*,2009 study which also showed 12% and 14% respectively.[7]

Among the parasitic agents protozoa like, *Acanthamoeba spp* was isolated in 6 patient (6.38%) and this was the only parasitic agent isolated and identified in infectious keratitis patients due to trauma.(Table 9)

Acanthamoeba keratitis was more common in agricultural workers and rural population and this may be due to greater

incidence of vegetative corneal injury among rural population engaged in Agriculture.

In this study corneal injury was the principal risk factor for fungal and *Acanthamoeba keratitis.* This study similar to M.Jayahar Bharthi *et al,* 2009 which also showed similar report.[7]Among the corneal injury with vegetative matter was the more often associated with fungal keratitis and injury with mud with Acanthamoeba keratitis.[99,107]

This study was similar to M.Jayahar Bharathi *et al,*[45] 2009, M.Sharma *et al,* 2000 studies which showed that 15% of corneal trauma was the predisposing factor for *Acanthamoeba keratitis*.[90]

Ocular infection due to Acanthamoeba was rare before two decades It was first reported in 1973 in both USA and UK (Bharathi *et al,* 2007).[5] In our country, first case of *Acanthamoeba keratitis* was reported in 1988 by Sharma *et al*,[90] (1990 b). Corneal infection from *Acanthamoeba* is believed to result from direct corneal contact with contaminated material or water. A high level of clinical suspicion and wet mount examination of specimens from infected tissue were essential to aid in rapid diagnosis of *Acanthamoeba keratitis* (Sharma *et al*, 1990 b).[90]

Acanthamoeba keratitis is a growing clinical problem in

developed as well as developing countries. Various Indian studies show prevalence rate of 1 to 4% among culture positive corneal ulcers. (Sharma *et al*,[90] 1990 b, 2000; Manikandan *et al*,[66] 2004; Devamani *et al*,[21] 1998). Most of the patient with *Acanthamoeba keratitis* (AK) belong to low socio economic group.

In developed countries, the single most important risk factor is wearing of contact lens. It is associated with 75% to 93% cases of Acanthamoeba keratitis (Jeanette *et al*,[46] 1989; Cherry *et al*,[18] 1998; Illingworth *et al*,[38,39] 1995). In developing countries, besides contact lens wearing, fall of dust particles, trauma due to vegetable matter, contact with contaminated water etc. have been found to be predominant risk factors of *Acanthamoeba keratitis* (Bharathi *et al*,[5] 2007; Sharma *et al*,[90] 1990 a,b, 2000; Manikandan *et al*,[66] 2004; Devamani *et al*,[21] 1998).

In this study all the 6 patients gave a definite history of trauma and immediate washing of the eye with stagnant dirty water.

Various studies have shown increasing prevalence of *Acanthamoeba keratitis* due to increased awareness of the clinical features and easy diagnostic techniques (Manikandan *et al*,[66] 2004; Jeanette *et al*,[46] 1989). A characteristic ring infiltrate of the central cornea was identified on examination of the cornea and 10% KOH

mount was done and *Acanthamoeba cysts* were identified. Similar clinical feature was described in many of the earlier studies (Bharathi *et al*,[5] 2007; Sharma *et al*,[90] l990b; Nicholson *et al*,[72] 1995).

In this study diagnosis of *Acanthamoeba keratitis* was suspected on observation of both cyst and motile trophozoites in normal saline wet mount examination of corneal ulcer scraping on first day. In most of the reported cases, a primary diagnosis of *Acanthamoeba keratitis* was not made and a delay in correct diagnosis ranged from 7 weeks to 12 months (Sharma *et al*,[90] 1990).

Specific drugs like Brolene and Polyhexamethylene biguainide were not available, so we used combination of Neosporin drops, Clotrimoxazole drops and Ketaconazole[43] tablet. Surprisingly response to these drugs was good. Corneal infection reduced within one week of therapy and ulcer completely healed within two months.[7] Corneal transplant may be indicated for medical failure and for impending or actual corneal perforation (Sharma *et al*,[19] 1990b).

The present study showed increasing prevalence of *Acanthamoeba keratitis* due to corneal trauma. Which is similar to Manikandan P *et al*, 2004.[66]

Anitbiotic susceptibility was performed for all the bacterial

isolate in this study by Kirby Bauer Disc diffusion method [44,70] which showed 94.11% sensitivity to Amikacin, 82.33% to Ciprofloxacin, and 79.41% to Cefatoxime, 61.76% to Ofloxacin, 76.47% to Cephalexin, 100% to Vancomycin.[53,79]

Amikacin was found to be the most effective antibiotic (94.11%) for the isolates in this study. This coincides with Savithri *et al*,[86,88] 1999 and Chien-fan-fong *et al*,[17] 2007 and Chalita M.R *et al*,[14] 2004 studies which also showed that Amikacin was found to be the effective drug against 93% - 95% of isolates.

Staphylococcus aureus exhibited 100% sensitivity to Amikacin, 77% to ciprofloxacin, 77% to Cephalexin, 88% to Cefatoxime and 77% to Oxacillin. In this study two isolates of *Staphylococcus aureus* were methicilin resistant. Both Methicillin resistant *Staphylococcus aureus* (MRSA) strain were 100% sensitive to Vancomycin. This study was similar to Sotozono C, *et al*, 2002. Methicilin being unstabable, oxacillin was used for studying the resistant pattern.

In this study *Pseudomonas aeruoginosa* was the second common bacterial isolates. In this study *Pseudomonas aeruoginosa* showed sensitivity pattern of 100% to Amikacin, 83% to Gentamicin, 66% to ciprofloxacin, 83% to Ofloxacin, 83% to

Cephalexin and 66% to Cefatoxime. Which was similar to Chein – Fen – Fong *et al*, [17] 2003 study.

In this study, Antifungal susceptibility was performed on 57 fungal isolates against Amphotericin B, Itraconazole, Fluconazole by Disc Diffusion method, Agar dilution method and Broth microdilution method (CLSI guidelines).[20]

Antifungal susceptibility pattern for the fungal isolates by Disc Diffusion method showed that 64% (9) *Aspergillus flavus*, 58% (7) *Aspergillus niger* isolates, 70% of (7) *Aspergillus fumigatus* isolates and 66% (8) *Fusarium* isolates, 50%(1) *Curvularia spp*, 33% (1) *Pencillium spp*, 50% (1) *Graphium spp* 50% (1) *Scopulariopsis spp* were sensitive to Amphotercin B (Table 12)

By Disc Diffusion method Itraconazole was sensitive in 78% (11) of *Aspergillus flavus*, 100% (12) *Aspergillus niger* isolates, 100% of (10) *Aspergillus fumigatus* isolates and 83% (10) *Fusarium* isolates, 50% (1) *Curvularia spp*, 60% (2) *pencillium spp*, 100% (2) *Graphium spp* 100% (2) *Scopulariopsis* spp were sensitive to Amphotercin B. All the fungal isolates were resistant to flucunazole by Disc Diffusion method. (Table 12)

For all fungal isolates MIC was perfomed by agar dilution

methods and Broth micro dilution methods for Amhotericin B, Itraconazole and Voriconazole antifungal drugs as per CLSI guidelines.[20]

By Agar dilution method 28/36 (84.84%) of *Aspergillus Spp*, 10/12 (83.83%) of *Fusarium Spp*, 100% of *Curvularia Spp*, *Graphium Spp* and *Scorpuloriopsis*. 2/3 (66.66%) of *Penicillium spp* showed sensitive range for Ampotericin B (MIC < 2 μg/ml)(Table 13)

For the fungal isolates MIC determination by broth microdilution method also showed that MIC range was comparable with Agar dilution method.[65] 84.74% of *Aspergillus spp* and 83% of *Fusarium* isolates, 100% of *Curvularia spp*, *Graphium spp*, *Scopulariopsis spp*, 66.60% of *Pencillium spp* showed sensitive range of Amphotercin B.

For Itraconaozole, (90.99%) of *Aspergillus spp* and 83% of *Fusarium* isolates, 100% of *Curvularia*, *Graphium spp*, *Scopularipsosis spp*, *Pencillium spp* showed MIC < 2 μg/ml. (Table 17)

For Voriconazole (96.96%) of *Aspergillus spp* and 91.66% of *Fusarium* isolates, 100% of *Curvularia spp*, *Graphium spp*, *Scopulariopsis spp*, *Pencillium spp* showed MIC < 2 μg/ml. (Table 18)

In this study Broth micro dilution method was found to be better than Agar dilution method and higher number of fungal isolates were found to be sensitive in Broth micro dilution method than the Agar dilution method.(Table 19)

10% Potassium hydroxide mount preparation used as screening test for rapid diagnosis of infectious keratitis case showed sensitivity 92.57% and 97.91% specificity (Table 20).

The sensitivity of 10% KOH mount and calcoflour stain in the diagnosis of *Acanthamoeba keratitis* was 92.99% and 100% respectively.

Prevention of Acanthamoeba keratitis by creating awareness about the predisposing factors among the public, is the prime responsibility of the clinicians and microbiologists.

SUMMARY

SUMMARY

1. A total of 150 patients with infectious keratitis due to trauma were included in the study, in which etilogical agent were identified and isolated in 94 cases. (62.66%)
2. Male predominance was seen in the study. (71.11%)
3. The age most commonly affected was between 51-60 years. (28.72%)
4. Incidence of infectious keratitis was more in the rural population than urban population. (64.66%) and majority of the patients were agricultural or manual labourers.
5. Majority of the isolates were fungal agents (57.44%) belonging to genus *Aspergillus spp* (63.15%) followed by *Fusarium spp* (21.05%) and *Pencillium spp*(5.26%)
6. Bacterial infectious keratitis were seen only in 32.97%. The predominant bacterial pathogen isolated was S*taphylococcus aureus* (26.47%) followed by *Pseudomonas aeruginosa*. (17.64%)
7. Infectitous keratitis due to parasitic etilology was 6.38%. A*canthamoeba spp* was the only parasitic agent isolated in this study

8. Trauma with vegetative matter was found to be the most common predisposing factor (47%) in the development of infectious keratitis.
9. 10% KOH mount was found to be very sensitive (92.59%) rapid screening test to diagnose fungal keratitis.
10. In anti fungal susceptibility by Disk diffusion Method showed, 80.7% of the fungal isolates were sensitive to Amphotericin B. 82% of the fungal isolates were sensitive to Itraconazole, 96% of the fungal isolates were sensitive to Voriconazole, and all the fungal isolates were resistant to Flucanazole by disk diffusion method.
11. In anti fungal susceptibility by Broth Micro dilution method showed, 82.45% of the fungal isolates were sensitive to Amphotericin B, 89.47% of the fungal isolates were sensitive to Itraconazole, 98.24% of the fungal isolates were sensitive to Voriconazole.
12. There was a good correlation between antifungal susceptibility results obtained by Broth microdilution method and Agar dilution method. In Broth micro dilution method more fungal isolates were sensitive than by Agar dilution method for Amphotericin B Itraconazole, and Voricanozole .

13. Majority of the Bacterial isolates were sensitive to amikacin. (94.11%) Two isolates of *S.aureus* were found to be methicillin resistant *Staphylococcus aureus* (MRSA) and among the *Staphylococcus epidermidis* two were found to be methicillin resistant Both the isolates were sensitive to Vancomycin (100%)
14. The sensitivity and specificity of 10% KOH mount was 92.59% and 97.91%) respectively for identification of fungal and parasitic keratitis.

CONCLUSION

CONCLUSION

Infectious keratitis is one of the most important cause of preventable blindness in the developing world. Infectious keratitis was predominant in males of rural background with vegetative matter induced ocular trauma as the major predisposing factor. A simple 10% KOH mount preparation was highly beneficial as rapid screening test for diagnosis. Among the isolates fungal keratitis were found to be more common than the bacterial and parasitic keratitis. *Aspergillus spp* and *Staphylococcus aureus* were the most common fungal and bacterial agents causing infectious keratitis. Among the parasitic agent, *Acanthamoeba spp* was the only parasitic agent isolated. From the present study, vital role of microbiological evaluation in the management of infectious keratitis is clearly evident, since the clinical features alone are not adequate to confirm infection. It is important to create awareness among people especially from rural background with regard to trauma as a major predisposing factor for Infectious keratitis. Present study show that diagnostic corneal scraping and culture (Gold standard) are mandatory in order to identify the causative organisms when Infectious keratitis is suspected and to choose

appropriate antimicrobial therapy. The present study showed that increasing prevalence of Acanthamoeba keratitis is seen among the non contact lens wearer. Prevention of Acanthamoeba keratitis by creating awareness about the predisposing factors among the public is the prime responsibility of the clinicians and microbiologists.

PROFORMA

Name : OP / IP No.

Age : Ward :

Sex : Address :

Occupation :

Presenting Complaints: **Duration :**

History of:

- Trauma – H/o. Injury, Type of Injury, Agent Injury (Physical / Chemical),
    - Any type of surgery and management
    - H/o. Occupation
- Prior Treatment
- Contact Lens wear
- Similar recurrent complaints
- Diabetes
- Hypertension

Clinical Examination:

- General Physical Examination

Built / Nourishment Pulse BP

- Ocular Examination
- Eye lid
- Conjunctiva
- Cornea – Position, Size of Ulcer, Margins, Slough, Satellite Lesions, Corneal sensation.Anterior Segment Examination –Pupils, HypopyonVision

Investigation

- Staining with 2% fluorescein
- Syringing of nasolacrimal duct
- Blood sugar

Microbiological Investigations

Direct Microscopic Examination

1) Mount

- Wet Mount

- KOH

2) Stains

- Gram stain

- Giemsa stain

- Lactophenol cotton blue stain

- Calcofluor white stain

- Modified - acid fast stain

3) Culture

Bacterial Culture

- Sheep Blood Agar – 5-20% CO_2 at 37^0C

- Chocolate Agar – 37^0C

- Thioglycollate Broth

- Brain heart infusion Agar

- Macconkey Agar

Fungal Culture

- Sabouraud's dextrose Agar – 25^0C & 37^0C

Parasitic Culture

- Agar Agar, Nonnutrient Agar Base, and coat the surface with bacteria. (E.Coli)

4) Parasitic Worms

- Macroscopic appearance and Mount examination

5) Antimicrobial susceptibility for bacterial and Fungal agent.

Bacterial – Disc diffusion method and broth dilution method

Fungal – Disc Diffusion, agar dilution and broth dilution method

A STUDY ON BACTERIAL, FUNGAL AND PARASITIC AGENT IN INFECTIOUS KERATITIS PATIENT DUE TO TRAUMA IN A TERTIARY CARE OPHTHALMIC HOSPITAL

CONSENT FORM:

STUDY TITLE:

I _____, hereby give consent to participate in the study conducted by Dr. C. Senthil Vadivu, Post Graduate at Institute of Microbiology, Madras Medical College, Rajiv Gandhi Government General Hospital, & Regional Institute of Ophthalmology Hospital, and to use my personal clinical data and the result of investigations for the purpose of analysis and to study the microbial profile and susceptibility pattern in traumatic infectious keratitis. I also give consent to give my corneal scrapping for further investigations. I also learn that there is no additional risk in this study. I also give my consent for my investigator to publish the data in any forum or journal.

Signature / Thumb Impression Place :
Of the Patient / relative Date :

Patient Name & Address :

Signature of the Investigator:

Signature of the Guide :

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MADICAL COLLEGE CHENNAI-3

Telephone No ; 0442530531
04425363970

CERTIFICATE OF APPROVAL

To
Dr.C.Senthil Vadivu
PG in MD Microbiology
Madras Medicals Collage, Channai-3

Dear Dr.C.Senthil Vadivu

The institutional Ethics committee of Madras Medical College reviewed and Discussed your application for approval of the proposal entitled "A study on bacterial, fungal and parasitic agents in infectious keratitis patients due to trauma in a tertiary care ophthalmic hospital, No. 20102011"

The following Members of Ethics Committee Were present in the. Meeting held on 20 .10.2011 conducted at Madras Medical college, Chennai -3,

1. Prof. S. K. Rajan. MD - Chairperson
2. Prof,A. Sundaram, MD - Member Secretary
3. Vice Principal ,Madras Medical Collage, Chennai -3
4. Prof R. Nandhini, MD
   Diractor,institute of Pharmacoiogy, MMC,Ch-3 - Member
5. Thiru AUlaganathan
   Administative Officer,MMC,Chennai – 3 - Layperson
6. Thiru. S.Goindasamy .,BABL - Layer
7. Thiru Arnold Soulina MA - Social Scientist
8. ProfShanta Raishankar - Member
   Prof ofNeuropathology,M.M.C,Chennai – 3

We approve the proposal to be conducted in its presented form

Sd/. Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information / informed consent and also asks to be provided a copy of the final report.

Member Secretary, Ethics Committee

APPENDIX

A. STAIN & REAGENTS:

1. 10% KOH :

Potassium hydroxide	:	10 g
Glycerol	:	10 ml
Distilled Water	:	80 ml

2. GRAM STAIN :

Methyl violet (2%)	:	10g methyl violet in 100 ml Absolute alcohol 1 lit. of distilled water (Primary Stain)
Gram Iodine	:	10g Iodine in 20g KI (fixative)
Acetone	:	Decolorising agent
Carbol fuchsin 1%	:	Counter stain

3. GIEMSA STAIN :

Giemsa Dye	-	1 gm
Methanol	-	85 ml
Glycerol	-	5 ml

4. Calcoflour White Stain :

This is a water soluble colourless textile dye fluorescent whitener. It selectively binds to chitin and cellulose of the fungal cell wall. It fluoresces light blue when exposed to UV light (346 – 365nm)

To the corneal scrapings in a slide, 1 drop of 0.1% calcoflour white with 0.1% Evan's blue and 1 drop of 10% KOH are added. A covership is placed over the specimen and examined under fluorescent microscope.

5. LACTOPHENOL COTTON BLUE:

For the staining and microscopic identification of fungi

Cotton Blue (Aniline Blue)	:	0.05 g
Phenol Crystals (C6H5O4)	:	20 g
Glycerol	:	40 ml
Lactic Acid (CH3CHOH COOH)	:	20 ml
Distilled Water	:	20 ml

MEDIA USED

1. Mac Conkey AGAR

Peptone	: 20 g
Sodium taurocholate	: 5 g
Distilled Water	: 1 lit
Agar	: 20 g
2% neutral red in 50% ethanol	: 3.5 ml
10% lactose solution	: 100 ml

Dissolve peptone and taurocholate in water by heating. Add agar and dissolve it in steamer. Adjust pH to 7.5. Add lactose and neutral red shake well and mix. Heat in free steam ($100^{o}C$) for 1 hour, then autoclave at $115^{o}C$ for 15 minutes.

2. BLOOD AGAR

Peptone	:	10 g
Nacl	:	5 g
Distilled Water	:	1 Ltr

Agar : 10 g

Dissolve ingredients in distilled water by boiling, by add 5% sheep blood (sterile) at 55^{o}C adjust pH to 7.4.

3. BRAIN HEART INFUSION BROTH:

Sodium citrate : 1 g

Sodium chloride : 4 g

Sodium phosphate : 5 g

Dextrose : 10 g

Peptone : 10 g

Brain heart infusion:

Brain infusion broth : 250 ml

Heart infusion broth : 750 ml

Sodium polyethonal sulphonate : 0.25 g

4. SABOURAUD'S DEXTROSE AGAR:

Dextrose : 20 g

Neo Peptone : 10 g

Agar	:	20 g
Distilled Water	:	1000 ml
pH	:	6.8 $\pm$ 0.2

Suspend the ingredients in water, dissolve by heating to a boil and dispense in approximately 20 ml amounts in cotton plugged 25x150 mm test tubes with antimicrobial agent (Gentamicin 20 mg) added after heating the medium and before autoclaving at 121^{o}C for no longer than 15 minutes. Slant was allowed to harden and refrigerated.

Note : Cycloheximide was not added to the media since it is known to inhibit ocular fungal pathogen.

5. POTATO DEXTROSE AGAR:

Potato	:	200g
Dextrose	:	20g
Agar	:	20g
Water	:	1 litre
pH	:	5.6 $\pm$ 0.2

Scrub, slice and boil potatoes in 100 ml of distilled water for one hour. Filter infusion through gauze and add agar and boil till it dissolve completely. Add dextrose and make upto one litre by adding distilled water. Sterilize by autoclaving at 15 pounds pressure at 115^{o}c for 30 minutes. Cool to 50^{o}c and approximately 20 ml into Petridishes.

6. MULLER HINTON AGAR:

Beef infusion	:	300 ml
Casein hydroxylate	:	17.5g
Starch	:	1.5g
Agar	:	10g
Distilled water	:	1 litre

pH : 7.4

Emulsify the starch in a small amount of cold water, pour into the beef infusion and add casein hydroxlysate and add agar. Make up the volume to 1 litre with distilled water. Dissolve the constituents by heating gently at 100^{o}c with agitation. Filter if necessary. Adjust the pH to 7.4 dispense in screw-capped bottles and sterilize by autoclaving the 121^{o}c for 20 minutes.

7. Choclate Agar :

Nutrient agar - 100ml

Lysed blood - 10 – 20ml

Melt the nutrient agar, cool to 50^0C add the lysed blood and pure plates.

8. Non-Nutrient Agar Media : (NN Media)

Agar - 1.5 gm

Sodium Chloride - 0.9 gm

Distilled water - 100 ml

Add agar and sodium chloride in distilled water mix well autoclave done and pour in plates.

9. RPMI 1640 MEDIUM:

Commercially purchased Rosewell Park Memorial Insititute (RPMI) 1640 media supplement with 0.3g of L-glutamate per litre without sodium bicarbonate (powder). Dissolve the powder in Nuclease free water. The medium was sterilized by filtering through a sterile membrane filter with a porosity of 0.22 microns. The pH was adjusted to 7.0. 3N – Morpholinopropane sulfonic acid (MOPS) buffer was used.

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MASTER CHART

S.No	Age	Sex	Bacterial	Fungal	Parasitic	Vegetative Mater	Dust	Thorn Prick	Cow's tail	Stone	Insect fall	Stick	Iron partical	KOK	Gram stain	Calco flour stain	NNAgar	Amikacin	Gentamicin	Ciprofloxacin	Cetataxime	Cotrimoxazole	Cephalexin	Vancomycin	Oxacillin	Amphotericin B (br,agar,disc)		Itraconazole (br,agar,disc)		Voriconazole (br,agar,disc)	
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101	62	F		A.nig		+								+												s	s	s	s	s	s
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131	56	M		Scop		+							+													s	s	s	s	s
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